#17

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re:	U.S. Patent No. 4,588,585	:
Issued:	May 13, 1986	:
Inventors:	David F. Mark Leo S. Lin Shi-da Yu Lu	:
For:	HUMAN RECOMBINANT CYSTEINE DEPLETED INTERFERON-β MUTEINS	:

TRANSMITTAL LETTER

Honorable Commissioner of
Patents and Trademarks
BOX PATENT EXTENSION
Washington, D.C. 20231

Sir:

Being filed herewith are the following papers:

1. Declaration Under 37 C.F.R. § 1.740(b);
2. Application for Extension of Patent Term Under 35 U.S.C. § 156;
3. Attachment A (copy of U.S. Patent No. 4,588,585);
4. Attachment B (brief description under 37 C.F.R. § 1.755); and
5. Attachment C - a copy of the Certificate of Correction;
6. Attachment D - a copy of the 1st maintenance fee paid - receipt;
7. A certified duplicate of all of the above.
8. Three courtesy working copies of 1-7.

SC13298 09/10/93 4588585 13-3402 130 111 1,000.00CH

Authorization is hereby granted to charge the fee of $1000 under 37 C.F.R. § 1.20(j) for fiing of an application for extension of the term of a patent to counsel's Deposit Account No. 13-3402. Two copies of this page are attached for this purpose. Authorization is also granted to charge any other fee which might be necessary in conjunction with this filing.

DATE: 9/9/93

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AJZ:dgg

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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re:	U.S. Patent No. 4,588,585	:
Issued:	May 13, 1986	:
Inventors:	David F. Mark Leo S. Lin Shi-Da Yu Lu	:
For:	HUMAN RECOMBINANT CYSTEINE DEPLETED INTERFERON-β MUTEINS	:

Honorable Commissioner of
Patents and Trademarks
BOX PATENT EXTENSION
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.740(b)

Sir:

The undersigned Attorney for Cetus Oncology Corporation which is the assignee of U.S.P. 4,588,585 and the Applicant submitting the attached Application for Extension of Patent Term under 35 U.S.C. 156 with regard to U.S. Patent No. 4,588,585 hereby declares as follows:

(1) THAT he is a patent attorney authorized to practice before the Patent and Trademark Office and has general authority from the owner to act on behalf of the assignee in patent matters;

(2) THAT he has reviewed and understands the contents of the application being submitted pursuant to 35 U.S.C. 156 and 37 C.F.R. 1.740;

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(3) THAT he believes the patent is subject to extension pursuant to 35 U.S.C. 156 and 37 C.F.R. 1.710.

(4) THAT he believes an extension of the length claimed is fully justified under 35 U.S.C. 156, and the applicable regulations.

(5) THAT he believes the patent for which the extension is being sought meets the conditions for extension of the term of a patent as set forth in 35 U.S.C. 156 and 37 C.F.R. 1.720.

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The undersigned hereby declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any extension of patent term issuing thereon.

Further declarant sayeth not.

Signed this 2nd day of September, 1993

Philip L. McGarrigle, Jr.
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Attorney for Applicant
Assistant Secretary and
Division Patent Counsel
Cetus Oncology Corporation
1400 Fifty-Third St.
Emeryville, California 94608
(510) 655-8730

PLM 9-2-93

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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re:	U.S. Patent No. 4,588,585	:
Issued:	May 13, 1986	:
Inventors:	David F. Mark Leo S. Lin Shi-Da Yu Lu	:
For:	HUMAN RECOMBINANT CYSTEINE DEPLETED INTERFERON-β MUTEINS	:

Commissioner of Patents and Trademarks
Box Patent Extension
Washington, D.C. 20231

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. 156

Sir:

Applicant, Cetus Oncology Corporation (former Cetus Corporation), a division of Chiron Corporation, organized and existing under and by virtue of the laws of the State of Delaware represents that it is the assignee of the entire interest in and to letters patent of the United States No. 4,588,585 granted to David F. Mark, Leo S. Lin and Shi-Da Yu Lu on May 13, 1986 for "HUMAN RECOMBINANT CYSTEINE DEPLETED INTERFERON-B MUTEINS." Said patent is a Division of USSN 564,224, December 20, 1983, Pat. No. 4,518,584 (now Re. 33,653), which is a C-I-P of USSN 486,162, April 15, 1983 abandoned, which is a C-I-P of USSN 435,134, October 19, 1982 abandoned.

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The assignment for said patent was recorded in the USPTO on September 25, 1992, Reel 6268, Frame 885.

Applicant acting through its duly authorized attorney, hereby submits this application for extension of patent term under 35 U.S.C. 156 by providing the following information required by the Rules promulgated by the U.S. Patent & Trademark Office (37 C.F.R. 1.710-1.785). For the convenience of the USPTO, the information presented in this application is in a format which follows the requirements of 37 C.F.R. 1.740.

(1) BETASERON®, the approved product, contains, as the active ingredient interferon beta-1b, whose chemical name is 17-L-serine interferon β^1 (human fibroblast reduced), also known as IFN-β_{ser17}, having the CAS registry number 90598-63-3 and the following structural formula:

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BETASERON®
INTERFERON BETA-1b
STRUCTURAL FORMULA

```
  *
H-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-Phe-
    2   3   4   5   6   7   8   9  10  11  12  13  14  15

Gln-Ser-Gln-Lys-Leu-Leu-Trp-Gln-Leu-Asn-Gly-Arg-Leu-Glu-Tyr-
 16  17  18  19  20  21  22  23  24  25  26  27  28  29  30

Cys-Leu-Lys-Asp-Arg-Met-Asn-Phe-Asp-Ile-Pro-Glu-Glu-Ile-Lys-
 31  32  33  34  35  36  37  38  39  40  41  42  43  44  45

Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-Ala-Ala-Leu-Thr-Ile-Tyr-
 46  47  48  49  50  51  52  53  54  55  56  57  58  59  60

Glu-Met-Leu-Gln-Asn-Ile-Phe-Ala-Ile-Phe-Arg-Gln-Asp-Ser-Ser-
 61  62  63  64  65  66  67  68  69  70  71  72  73  74  75

Ser-Thr-Gly-Trp-Asn-Glu-Thr-Ile-Val-Glu-Asn-Leu-Leu-Ala-Asn-
 76  77  78  79  80  81  82  83  84  85  86  87  88  89  90

Val-Tyr-His-Gln-Ile-Asn-His-Leu-Lys-Thr-Val-Leu-Glu-Glu-Lys-
 91  92  93  94  95  96  97  98  99 100 101 102 103 104 105

Leu-Glu-Lys-Glu-Asp-Phe-Thr-Arg-Gly-Lys-Leu-Met-Ser-Ser-Leu-
106 107 108 109 110 111 112 113 114 115 116 117 118 119 120

His-Leu-Lys-Arg-Tyr-Tyr-Gly-Arg-Ile-Leu-His-Tyr-Leu-Lys-Ala-
121 122 123 124 125 126 127 128 129 130 131 132 133 134 135

Lys-Glu-Tyr-Ser-His-Cys-Ala-Trp-Thr-Ile-Val-Arg-Val-Glu-Ile-
136 137 138 139 140 141 142 143 144 145 146 147 148 149 150

Leu-Arg-Asn-Phe-Tyr-Phe-Ile-Asn-Arg-Leu-Thr-Gly-Tyr-Leu-Arg-
151 152 153 154 155 156 157 158 159 160 161 162 163 164 165

Asn-OH
166
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* Numbered in accordance with native interferon-β (the native 1-met is cleaved off in the production of this protein in E. Coli).

(2) The approved product, BETASERON® was subject to regulatory review under the Federal Food, Drug and Cosmetic Act Section 505 (21 U.S.C. 355).

(3) The approved product, BETASERON® received permission for commercial marketing or use under Section 505 of the Federal Food, Drug and Cosmetic Act (21 U.S.C. 355) on July 23, 1993.

(4) The only active ingredient in BETASERON® is interferon beta-1b which has not been approved for commercial marketing or use under Section 505 of the Federal Food, Drug and Cosmetic Act, any other section thereof or any other Federal law prior to the approval of PLA 92-0495 by the Food and Drug Administration.

(5) This application for extension of patent term under 35 U.S.C. 156 is being submitted within the 60 day period pursuant to 37 C.F.R. 1.720(f) which period will expire September 21, 1993.

(6) The complete identification of the patent for which extension is being sought is as follows:

Inventors:	David F. Mark, Leo S. Lin & Shi-Da Yu Lu
Patent Number:	4,588,585
Issue Date:	May 13, 1986
Expiration Date:	May 13, 2003

(7) See Attachment A for a complete copy of the patent identified in paragraph (6) hereof.

(8) No disclaimer or re-examination certificate has issued with regard to U.S.P. 4,588,585. A Certificate of Correction (see Attachment C) was issued on May 10, 1988 by the USPTO in order to add a claim for priority under 35 U.S.C. 119 of Irish Patent application 2380/83 filed October 10, 1983. A 1st maintenance fee became due November 13, 1989 and was paid November 7, 1989 (see Attachment D). A second maintenance fee is due November 13, 1993.

(9) U.S. Patent 4,588,585 claims the approved product. Specifically the active ingredient interferon beta-1b per-se is claimed in claims 1, 2, 3, 4, 5, and 6 which follow and a therapeutic composition comprising such active ingredient is claimed in claim 7 which also follows.

CLAIMS

-1-

Recombinant, synthetic human interferon-β mutein, wherein the cysteine at position 17, numbered in accordance with native interferon-β, is deleted or replaced by a neutral amino acid, and wherein said mutein exhibits biological activity of native, human interferon-β.

The synthetic mutein of claim 1 wherein said cysteine residue is replaced by an amino acid selected from the group consisting of serine, threonine, glycine, alanine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan or methionine.

-3-

The synthetic mutein of claim 1 wherein said cysteine residue has been replaced by an amino acid selected from the group consisting of serine or threonine.

-4-

The synthetic mutein of claim 1 wherein the mutein is unglycosylated.

-5-

Biologically active IFN-β_{ser17}.

-6-

IFN-β_{ser17} as represented by the amino acid sequence represented in Fig. 10.

-7-

A therapeutic composition having IFN-β activity comprising a therapeutically effective amount of the synthetic mutein of claims, 1, 2, 3, 4, 5, or 6 admixed with a pharmaceutically acceptable carrier medium.

In addition, claims 8-10 of the patent which follow, cover certain methods of using the approved product.

-8-

A method of regulating cell growth in a patient comprising administering to said patient a cell growth regulating amount of the synthetic mutein of claims 1, 2, 3, 4, 5 or 6.

A method of treating a patients for viral disease comprising administering to said patient a viral disease inhibiting amount of the synthetic mutein of claims 1, 2, 3, 4, 5 or 6.

-10-

A method of stimulating natural killer cell activity in a patient comprising administering to said patient a natural killer cell stimulating amount of the synthetic mutein of claims 1, 2, 3, 4, 5, or 6.

(10) The relevant dates and information pursuant to 35 U.S.C. 156(g) to enable the Secretary of Health & Human Services to determine the applicable regulatory review period are as follows:

(i) The Investigational New Drug Application (IND-1846) Protocol TB01-16486 for Multiple Sclerosis was filed April 4, 1986. The Product License Application (PLA-92-0495) for BETASERON® (Interferon beta-1b) was submitted to the FDA on June 16, 1992. The FDA issued said Product License on July 23, 1993.

NOTE: An IND for Betaseron® was initially filed on April 15, 1983 for indications other than multiple sclerosis. Only the multiple sclerosis indication has been approved by FDA.

(11) A brief description of the significant activities (with dates) undertaken by Triton Biosciences, Inc. (now known as Berlex Biosciences, a division of Berlex Laboratories, Inc.), Berlex Biosciences, Berlex Laboratories Inc., (the latter being the marketing applicant), Cetus (now Cetus Oncology Corporation, a division of Chiron Corporation), and Chiron Corporation, (the latter being the manufacturing applicant), during the applicable regulatory review period is attached hereto as "Attachment B" and is a chronological synopsis of the major communications between Applicants and the FDA from April 4, 1986 to July 23, 1993.

(12) Applicant is of the opinion that U.S. Patent 4,588,585 is eligible for extension under 35 U.S.C. 156 because it satisfies all the requirements for such extension as follows:

(a) 35 U.S.C. 156(a); 37 C.F.R. 1.720(a)

U.S. Patent 4,588,585 claims a product as defined in 37 C.F.R. 1.710.

(b) 35 U.S.C. 156(a)(1); 37 C.F.R. 1.720(g)

The term of U.S. Patent 4,588,585 has not expired before submission of this application.

(c) 35 U.S.C. 156(a)(2); 37 C.F.R. 1.720(b)

The term of U.S. Patent 4,588,585 has never been extended.

(d) 35 U.S.C. 156(a)(3); 37 C.F.R. 1.730

The application for extension is submitted by the authorized agent or the owner of record in accordance with the requirements of 35 U.S.C. 156(d) and the Rules of the U.S. Patent & Trademark Office.

(e) 35 U.S.C. 156(a)(4); C.F.R. 1.720(d)

The product BETASERON® has been subjected to a regulatory review period as defined in 35 U.S.C. 156(g) before its commercial marketing or use.

(f) 35 U.S.C. 156(a)(5)(A); 37 C.F.R. 1.720(e)(1)

The commercial marketing or use of the product BETASERON® after the regulatory review period is the first permitted commercial marketing or use of the product under the provision of the Federal Food, Drug and Cosmetics Act (21 U.S.C. 355) under which such regulatory review period occurred.

(g) 35 U.S.C. 156(c)(4); 37 C.F.R. 1.720(h)

No other patent has been extended for the same regulatory review period for the product BETASERON®.

(h) 35 U.S.C. 156(d)(1); 37 C.F.R. 1.720(f)

The application is submitted within the permitted 60 day period beginning on the date the product first received permission for commercial marketing or use.

(i) The length of extension of the patent term of U.S.P. 4,588,585 claimed by application is 1500 days. The length of extension was determined pursuant to 37 C.F.R. 1.775 as follows:

(i) The regulatory review period under 35 U.S.C. 156 (g)(1)(B) began on April 4, 1986 and ended on July 23, 1993 which is a total of 2667 days or 7.31 years which is the sum of (ii) & (iii) below.

(ii) The period of review under 35 U.S.C. 156(g)(1)(B)(i), the "Testing Period" began on April 4, 1986 and ended on June 16, 1992 which is 2265 days or 6.21 years.

(iii) The period of review under 35 U.S.C. 156(g)(1)(B)(ii), the "Application Period" began on June 16, 1992 and ended July 23, 1993 which is 402 days or 1.1 years.

(j) The regulatory review period upon which the period of extension is calculated is the entire regulatory review period as determined in sub-paragraph 12(i)(i) (2667 days) less.

(i) The number of days in the regulatory review period which were on or before the date on which the patent issued (May 13, 1986) which is 39 days, and

(ii) The number of days during which applicant did not act with due diligence which is zero (0) days, and

(iii) One-half the number of days determined in sub-paragraph 12(i)(ii) after subtracting therefrom the number of days in sub-paragraphs 12(j)(I) and j(ii) or 1113 days. This total is now 1554 days.

(k) The number of days as determined in sub-paragraph 12(j)(iii) (1554 days) when added to the original term of the patent would result in the date September 15, 2007.

(l) Fourteen (14) years, when added to the date of the NDA approval (July 23, 1993) would result in the date July 23, 2007.

(m) The earliest date as determined in paragraphs 12(k) and 12(l) is July 23, 2007.

(n) The issuance of the original patent was May 13, 1986 which is after September 24, 1984. Five (5) years added to the original expiration date would be May 13, 2008.

(o) The earlier date as determined in paragraph (m) and (n) is July 23, 2007.

Therefore, the length of extension of patent term claimed by applicant is 1500 days.

(13) Applicant acknowledges a duty to disclose to the Commissioner of Patents and Trademarks and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought.

(14) The prescribed fee for receiving and acting upon this application is to be charged to Deposit Account No. 13-3402, as authorized in the attached transmittal letter, which is submitted in triplicate.

(15) All inquiries and correspondence relating to this application are to be directed to Anthony J. Zelano, Esq., Millen, White, Zelano & Branigan,

P.C., Arlington Courthouse Plaza I, Suite 1400, 2200 Clarendon Boulevard, Arlington, VA 22201, (703) 243-6333.

(16) A duplicate of these application papers, certified as such, is being submitted herewith.

(17) The requisite Declaration pursuant to rule 37 C.F.R. 1.740(b) is attached hereto.

Respectfully submitted,

PLM 9-293

Philip L. McGarrigle, Jr.
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Attorney for Applicant
Assistant Secretary and
Division Patent Counsel
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ATTACHMENT A

United States Patent [19]

Mark et al.

[11] Patent Number: 4,588,585

[45] Date of Patent: May 13, 1986

[54] HUMAN RECOMBINANT CYSTEINE DEPLETED INTERFERON-β MUTEINS

[75] Inventors: David F. Mark, Danville; Leo S. Lin, Fremont; Shi-Da Yu Lu, Oakland, all of Calif.

[73] Assignee: Cetus Corporation, Emeryville, Calif.

[21] Appl. No.: 655,897

[22] Filed: Sep. 28, 1984

Related U.S. Application Data

[60] Division of Ser. No. 564,224, Dec. 20, 1983, Pat. No. 4,518,584, which is a continuation-in-part of Ser. No. 486,162, Apr. 15, 1983, abandoned, which is a continuation-in-part of Ser. No. 435,154, Oct. 19, 1982, abandoned.

[51] Int. Cl.[4] A61K 45/02; C07K 13/00; C07K 15/26; C12P 21/00

[52] U.S. Cl. 424/85; 435/172.3; 435/68; 530/351

[58] Field of Search ..... 260/112 R, 112.5 R; 424/85; 435/172.3, 68

[56] References Cited

U.S. PATENT DOCUMENTS

4,414,150 11/1983 Goeddel 424/85

FOREIGN PATENT DOCUMENTS

28033 5/1981 European Pat. Off. .
42246 12/1981 European Pat. Off. .
41313 12/1981 European Pat. Off. .
131816 1/1985 European Pat. Off. .

OTHER PUBLICATIONS

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Glossary of Genetics and Cytogenetics, 4th ed., p. 381, Springer–Verlag (1976).
Taniguchi, T. et al., *Gene* (1980) 10:11–15.
Taniguchi, T. et al., *Nature* (1980) 285:547–549.
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Smith, M. and Gillam, S., *Genetic Engineering* Principles and Methods, Plenum Press (1981) 3:1–32.
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Taniguchi, T. et al., *PNAS* (1980), 77 (9) 5230–5233.
Allen G. et al., *Nature* (1980) 287, 408–410.
Levy, W. et al., *PNAS* (1981), 78 (10) 6186–6190.
Knight, S. E. et al., *Journal of Interferon Research*, (1982), 2 (3) 421–429.

Primary Examiner—Blondel Hazel
Attorney, Agent, or Firm—Albert P. Halluin; Leona L. Lauder; Thomas E. Ciotti

[57] ABSTRACT

Muteins of biologically active proteins such as IFN-β and IL-2 in which cysteine residues that are not essential to biological activity have been deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bridge formation. These muteins are made via bacterial expression of mutant genes that encode the muteins that have been synthesized from the genes for the parent proteins by oligonucleotide-directed mutagenesis.

10 Claims, 19 Drawing Figures

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                5                  10                  15                  20
MetSerTyrAsnLeu LeuGlyPheLeuGln ArgSerSerAsnPhe GlnCysGlnLysLeu
               25                  30                  35                  40
LeuTrpGlnLeuAsn GlyArgLeuGluTyr CysLeuLysAspArg MetAsnPheAspIle
               45                  50                  55                  60
ProGluGluIleLys GlnLeuGlnGlnPhe GlnLysGluAspAla AlaLeuThrIleTyr
               65                  70                  75                  80
GluMetLeuGlnAsn IlePheAlaIlePhe ArgGlnAspSerSer SerThrGlyTrpAsn
               85                  90                  95                 100
GluThrIleValGlu AsnLeuLeuAlaAsn ValTyrHisGlnIle AsnHisLeuLysThr
              105                 110                 115                 120
ValLeuGluGluLys LeuGluLysGluAsp PheThrArgGlyLys LeuMetSerSerLeu
              125                 130                 135                 140
HisLeuLysArgTyr TyrGlyArgIleLeu HisTyrLeuLysAla LysGluTyrSerHis
              145                 150                 155                 160
CysAlaTrpThrIle ValArgValGluIle LeuAgAsnPheTyr PheIleAsnArgLeu
              165                 170                 175                 180
ThrGlyTyrLeuArg Asn---
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FIG. 1

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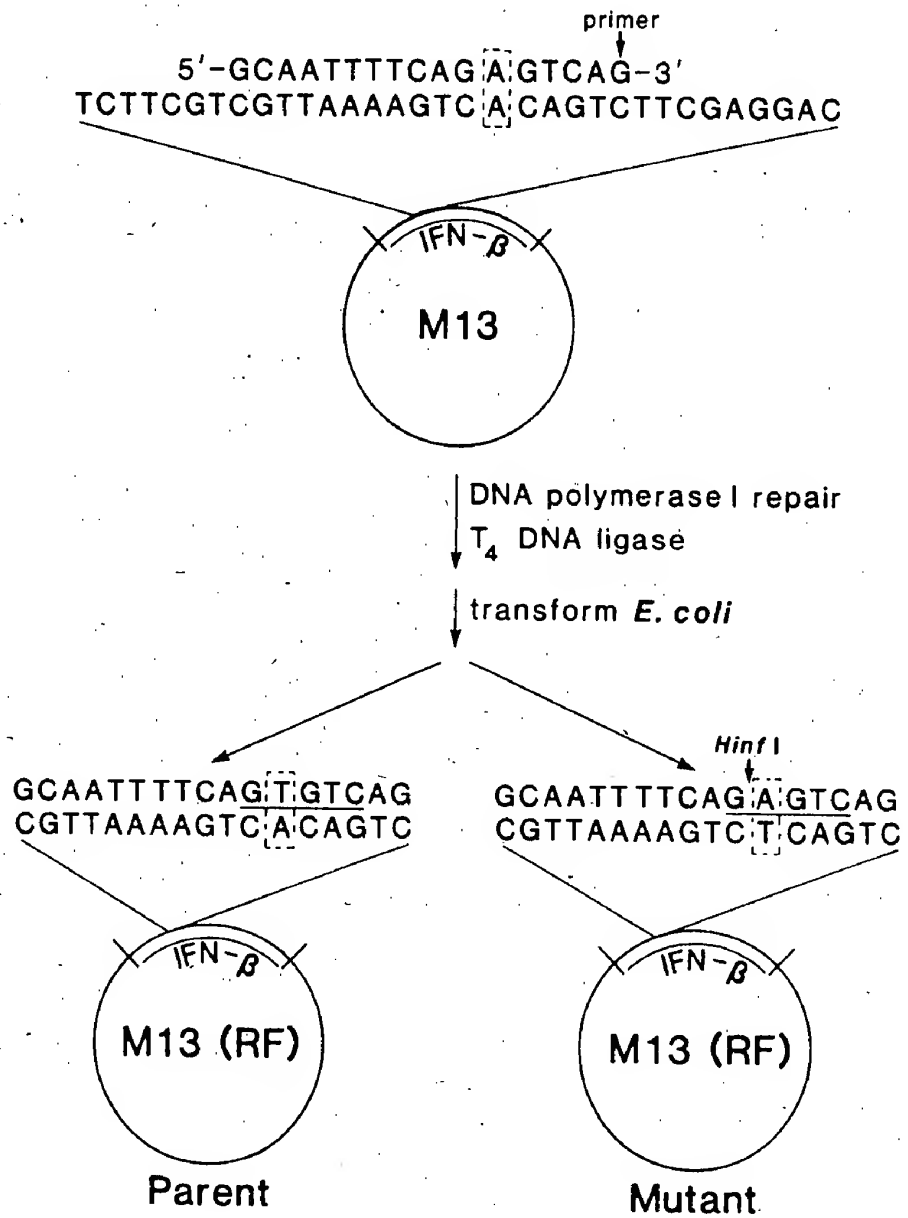

primer
5'-GCAATTTTCAGAGTCAG-3'
TCTTCGTCGTTAAAAGTCACAGTCTTCGAGGAC
IFN-β
M13
DNA polymerase I repair
T4 DNA ligase
transform E. coli
Hinf I
GCAATTTTCAGTGTCAG
CGTTAAAAGTCACAGTC
GCAATTTTCAGAGTCAG
CGTTAAAAGTCTCAGTC
IFN-β
M13 (RF)
Parent
IFN-β
M13 (RF)
Mutant

FIG. 2

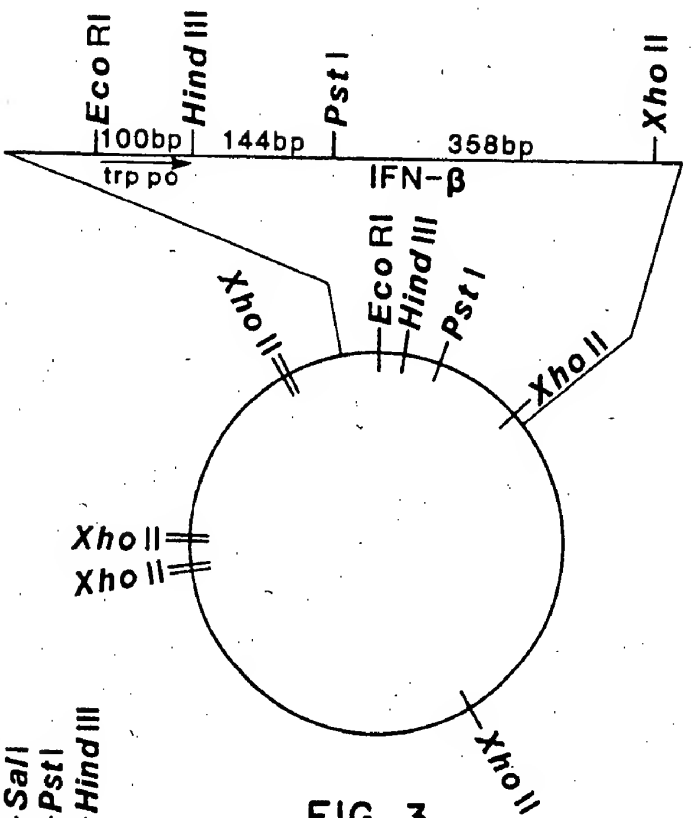

Eco RI
Hind III
Pst I
Xho II
100bp
144bp
358bp
trp po
IFN-β
Xho II
Eco RI
Hind III
Pst I
Xho II
Xho II
Xho II
Xho II

FIG. 3

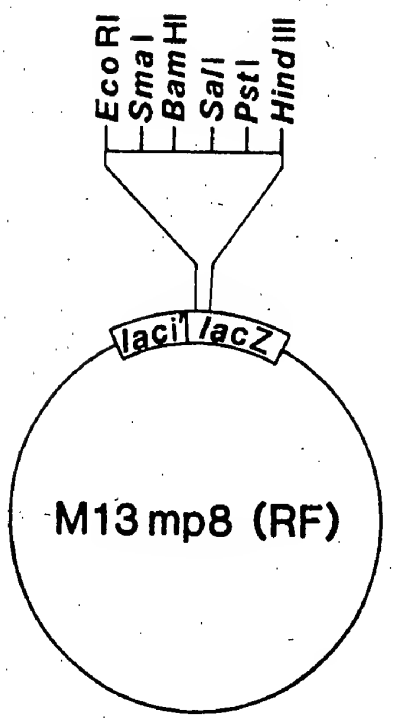

Eco RI
Sma I
Bam HI
Sal I
Pst I
Hind III
lacI
lacZ
M13 mp8 (RF)

FIG. 4

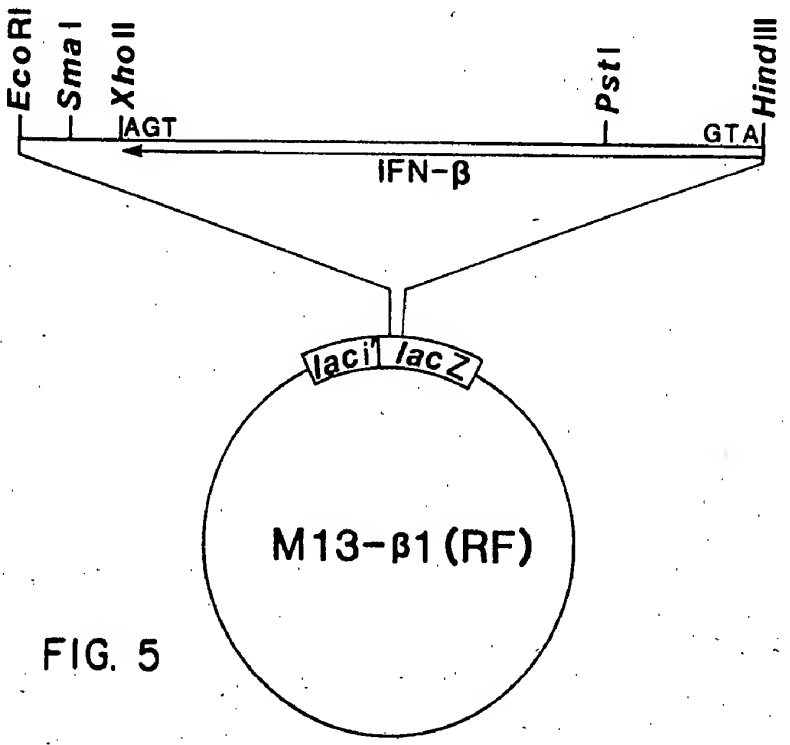

EcoRI
Sma I
Xho II
AGT
Pst I
GTA
Hind III
IFN-β
lac i
lac Z
M13-β1 (RF)

FIG. 5

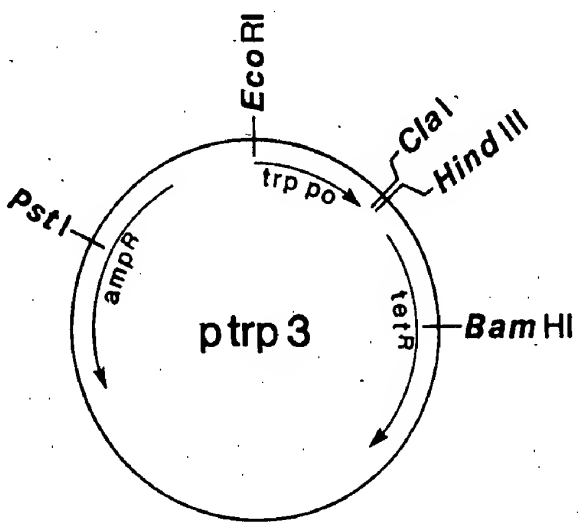
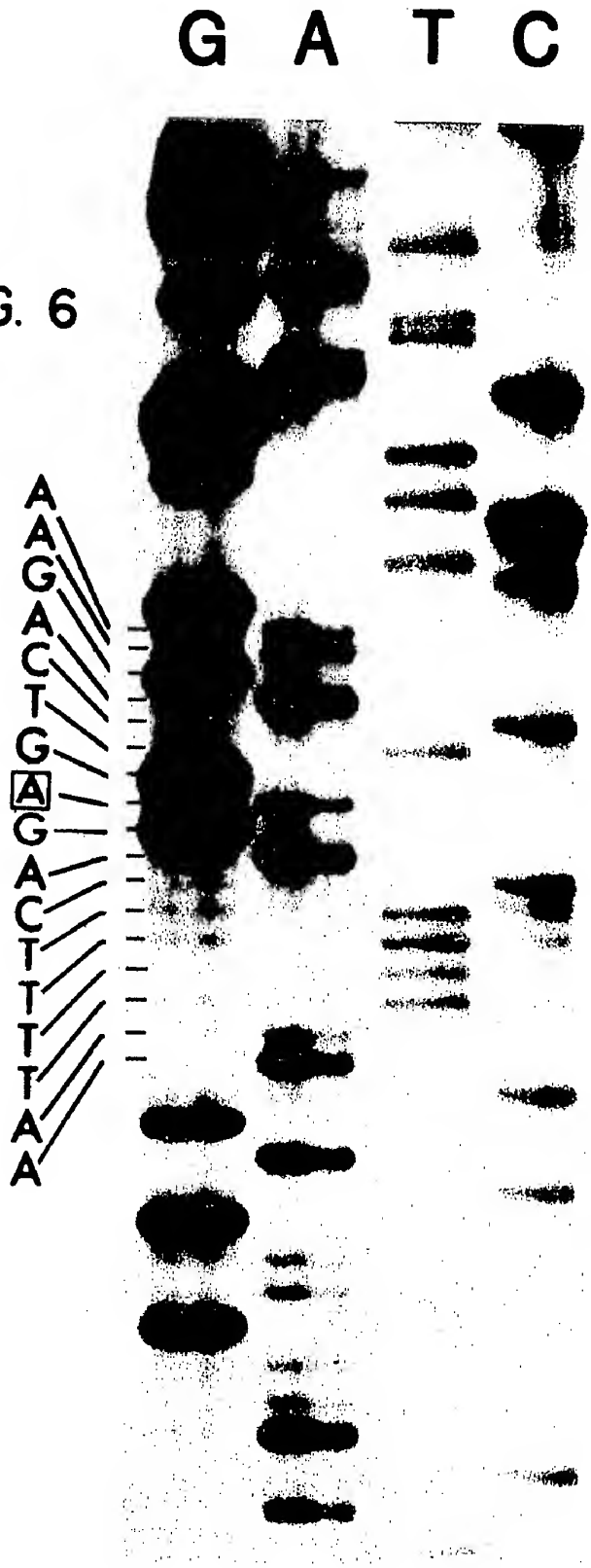

EcoRI
Cla I
Hind III
trp po
Pst I
ampR
ptrp3
tetR
Bam HI

FIG. 7

FIG. 6



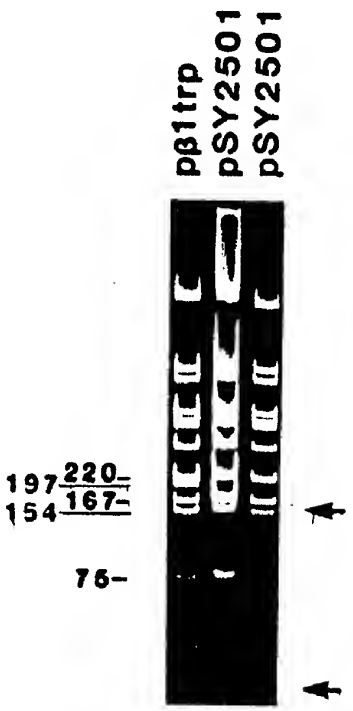

pβ1trp
pSY2501
pSY2501
197
154
220
167
76

FIG. 8a

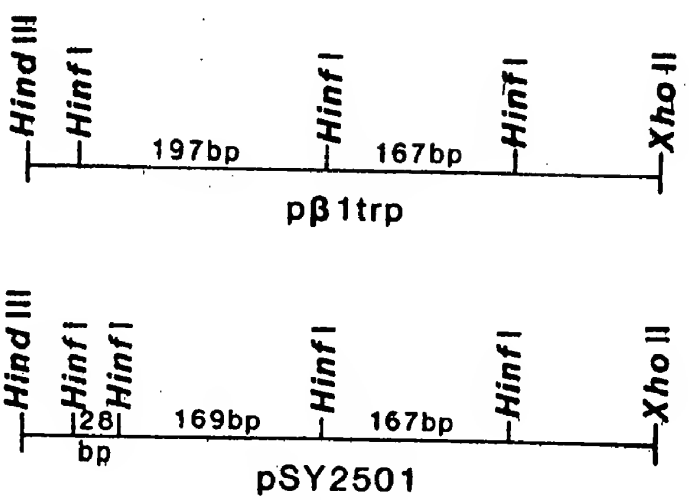

Hind III
Hinf I
Hinf I
Hinf I
Xho II
197bp
167bp
pβ1trp
Hind III
Hinf I
Hinf I
Hinf I
Hinf I
Xho II
28 bp
169bp
167bp
pSY2501

FIG. 8b

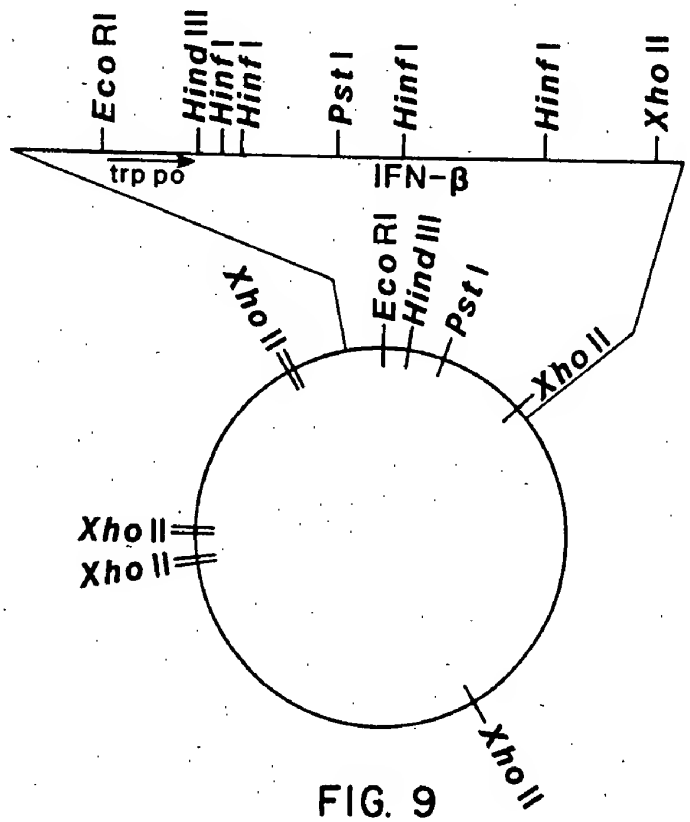

Eco RI
Hind III
Hinf I
Hinf I
Pst I
Hinf I
Hinf I
Xho II
trp po
IFN-β
Xho II
Eco RI
Hind III
Pst I
Xho II
Xho II
Xho II
Xho II

FIG. 9

IFN-B CYS TO SER CHANGE AT AMINO ACID 17

```
1                                                                  17
ATG AGC TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG AGT CAG AAG CTC
met ser tyr asn leu leu gly phe leu gln arg ser ser asn phe gln ser gln lys leu

61
CTG TGG CAA TTG AAT GGG AGG CTT GAA TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC
leu trp gln leu asn gly arg leu glu tyr cys leu lys asp arg met asn phe asp ile

121
CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT
pro glu glu ile lys gln leu gln gln phe gln lys glu asp ala ala leu thr ile tyr

181
GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG AAT
glu met leu gln asn ile phe ala ile phe arg gln asp ser ser ser thr gly trp asn

241
GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA
glu thr ile val glu asn leu leu ala asn val tyr his gln ile asn his leu lys thr

301
GTC CTG GAA GAA AAA CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG
val leu glu glu lys leu glu lys glu asp phe thr arg gly lys leu met ser ser leu

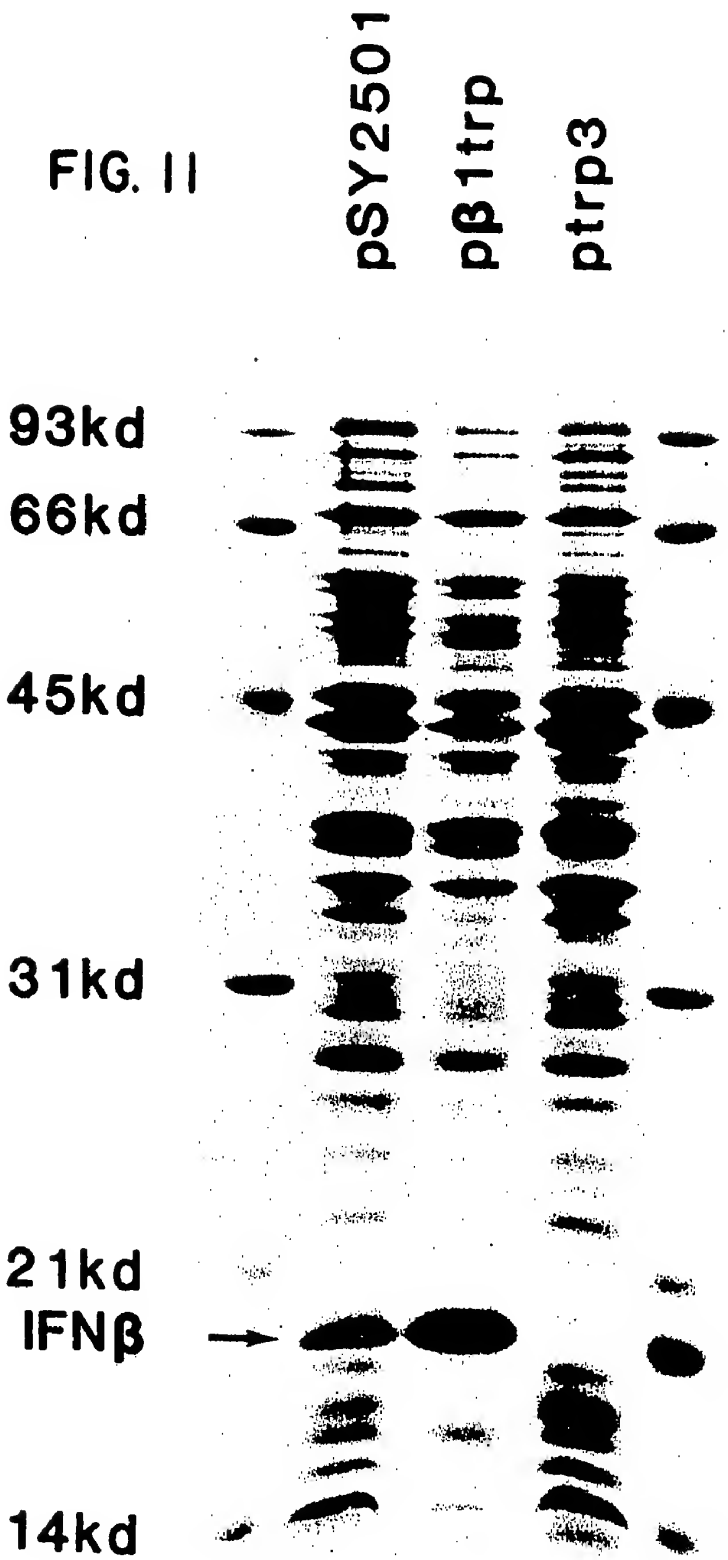
361
CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC
his leu lys arg tyr tyr gly arg ile leu his tyr leu lys ala lys glu tyr ser his

421
TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT
cys ala trp thr ile val arg val glu ile leu arg asn phe tyr phe ile asn arg leu

481
ACA GGT TAC CTC CGA AAC TGA AGA TC
thr gly tyr leu arg asn *** 
```

FIG. 10

FIG. 11



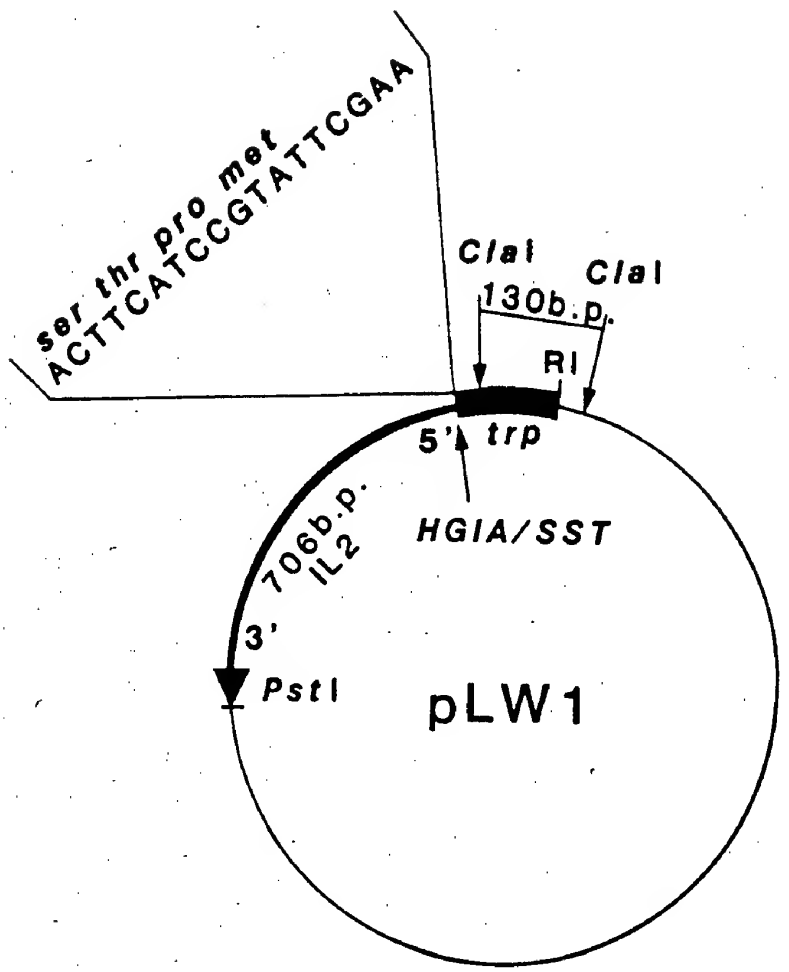

ser thr pro met
ACTTCATCCGTATTCGAA
ClaI
ClaI
130b.p.
RI
5'
trp
HGIA/SST
706b.p.
IL2
3'
PstI
pLW1

FIG. 12

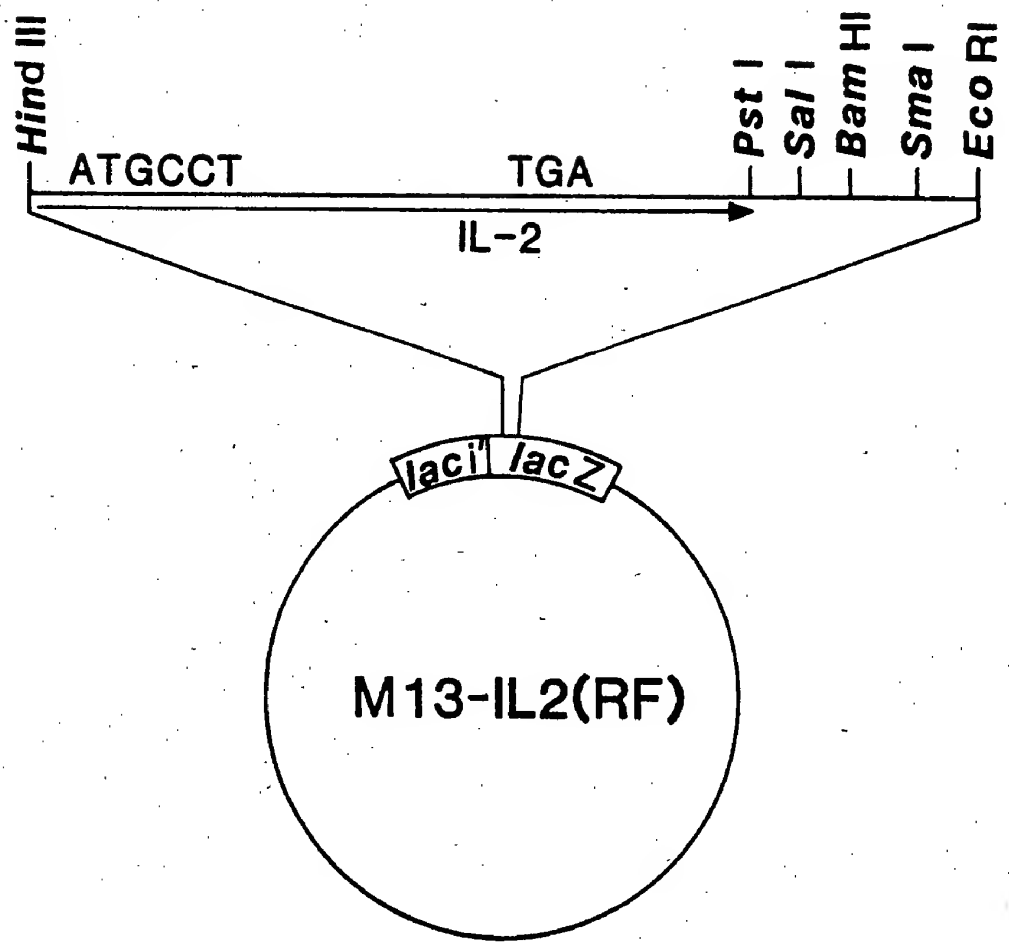

Hind III
ATGCCT
TGA
Pst I
Sal I
Bam HI
Sma I
Eco RI
IL-2
lacI'
lacZ
M13-IL2(RF)

FIG. 13

320

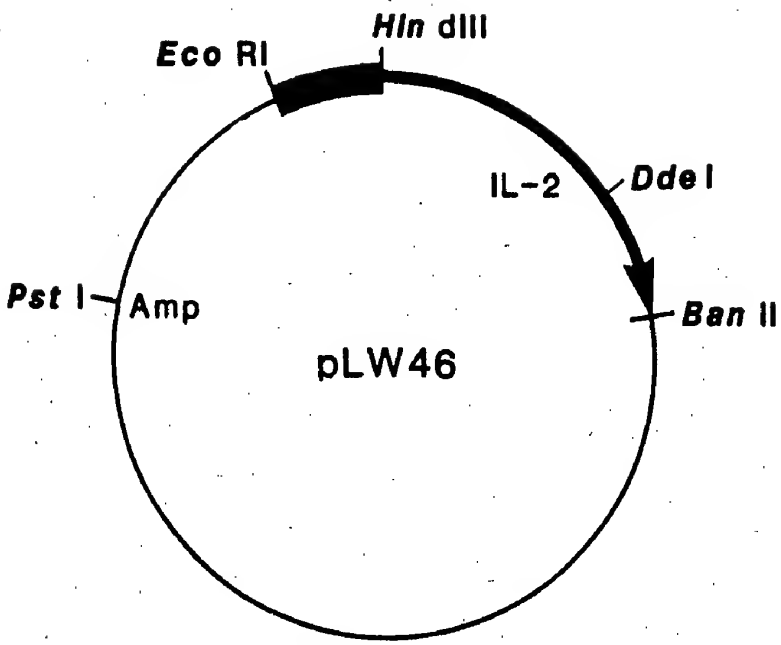


FIG. 14

```
        10          20          30          40          50          60
ATGCCTACTT CAAGTTCTAC AAAGAAAACA CAGCTACAAC TGGAGCATTT ACTGCTGGAT
        70          80          90         100         110         120
TTACAGATGA TTTTGAATGG AATTAATAAT TACAAGAATC CCAAACTCAC CAGGATGCTC
       130         140         150         160         170         180
ACATTTAAGT TTTACATGCC CAAGAAGGCC ACAGAACTGA AACATCTTCA GTGTCTAGAA
       190         200         210         220         230         240
GAAGAACTCA AACCTCTGGA GGAAGTGCTA AATTTAGCTC AAAGCAAAAA CTTTCACTTA
       250         260         270         280         290         300
AGACCCAGGG ACTTAATCAG CAATATCAAC GTAATAGTTC TGGAACTAAA GGGATCTGAA
       310         320         330         340         350         360
ACAACATTCA TGTGTGAATA TGCTGATGAG ACAGCAACCA TTGTAGAATT TCTGAACAGA
       370         380         390         400         410         420
TGGATTACCT TTTCTCAGAG CATCATCTCA ACACTGACTT GA
```

FIG. 15a

```
                5                  10                  15                  20
MetProThrSerSer SerThrLysLysThr GlnLeuGlnLeuGlu HisLeuLeuLeuAsp
               25                  30                  35                  40
LeuGlnMetIleLeu AsnGlyIleAsnAsn TyrLysAsnProLys LeuThrArgMetLeu
               45                  50                  55                  60
ThrPheLysPheTyr MetProLysLysAla ThrGluLeuLysHis LeuGlnCysLeuGlu
               65                  70                  75                  80
GluGluLeuLysPro LeuGluGluValLeu AsnLeuAlaGlnSer LysAsnPheHisLeu
               85                  90                  95                 100
ArgProArgAspLeu IleSerAsnIleAsn ValIleValLeuGlu LeuLysGlySerGlu
              105                 110                 115                 120
ThrThrPheMetCys GluTyrAlaAspGlu ThrAlaThrIleVal GluPheLeuAsnArg
              125                 130                 135                 140
TrpIleThrPheSer GlnSerIleIleSer ThrLeuThr---
```

FIG. 15b

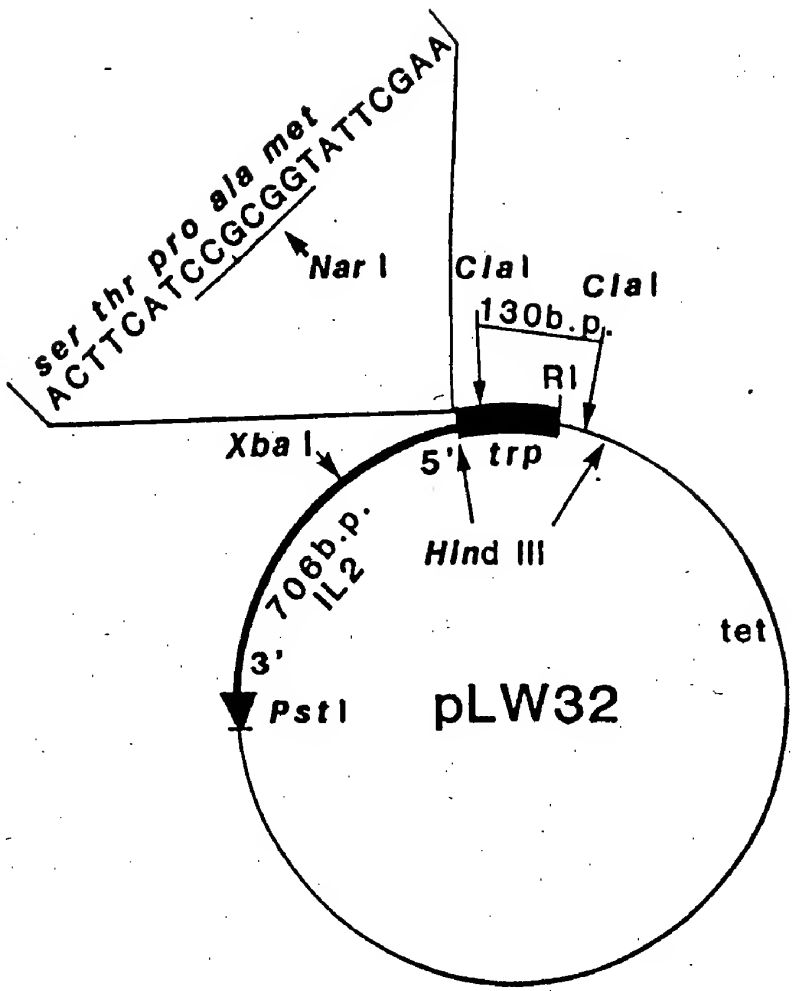

ser thr pro ala met
ACTTCATCCGCGGTATTCGAA
Nar I
Cla I
Cla I
130b.p.
RI
Xba I
5'
trp
706b.p.
IL2
Hind III
tet
3'
Pst I
pLW32

FIG. 16

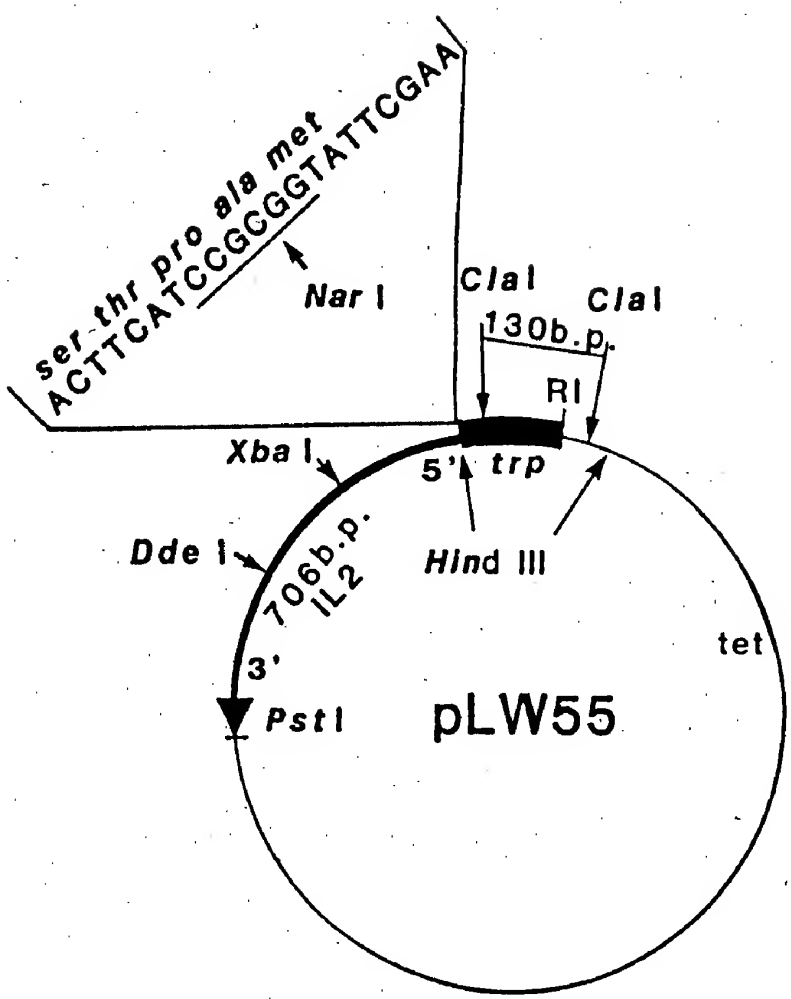

ser thr pro ala met
ACTTCATCCGGGGTATTCGAA
Nar I
ClaI
ClaI
130b.p.
RI
Xba I
5'
trp
Dde I
706b.p.
IL2
Hind III
3'
Pst I
pLW55
tet

FIG. 17

HUMAN RECOMBINANT CYSTEINE DEPLETED INTERFERON-β MUTEINS

CROSS-REFERENCE TO RELATED APPLICATION

This application is a division of U.S. Ser. No. 564,224, filed Dec. 20, 1983, now U.S. Pat. No. 4,518,584, which in turn is a continuation-in-part of U.S. Ser. No. 486,162 filed Apr. 15, 1983 now abandoned, which is a continuation-in-part of U.S. Ser. No. 435,154 filed Oct. 19, 1982.

DESCRIPTION

1. Technical Field

This invention is in the general area of recombinant DNA technology. More specifically it relates to mutationally altered biologically active proteins that differ from their parent analogs by one or more substitutions/deletions of cysteine residues.

2. Background Art

Biologically active proteins that are microbially produced via recombinant DNA (rDNA) technology may contain cysteine residues that are nonessential to their activity but are free to form undesirable intermolecular or intramolecular links. One such protein is microbially produced human beta interferon (IFN-β). In the course of the preparation of IFN-β by rDNA techniques, it has been observed that dimers and oligomers of microbially produced IFN-β are formed in *E.coli* extracts containing high concentrations of IFN-β. This multimer formation renders purification and separation of IFN-β very laborious and time-consuming and necessitates several additional steps in purification and isolation procedures such as reducing the protein during purification and reoxidizing it to restore it to its original conformation, thereby increasing the possibility of incorrect disulfide bond formation. Furthermore, microbially produced IFN-β has also been found to exhibit consistently low specific activity due perhaps to the formation of multimers or of random intramolecular disulfide bridges. It would be desirable, therefore, to be able to alter microbially produced biologically active proteins such as IFN-β in a manner that does not affect their activity adversely but reduces or eliminates their ability to form intermolecular crosslinks or intramolecular bonds that cause the protein to adopt an undesirable tertiary structure (e.g., a conformation that reduces the activity of the protein).

The present invention is directed to producing by directed mutagenesis techniques mutationally altered biologically active proteins (such proteins are called "muteins", *Glossary of Genetics and Cytogenetics,* 4th Ed, p 381, Springer-Verlag (1976)) that retain the activity of their parent analogs but lack the ability to form intermolecular links or undesirable intramolecular disulfide bonds. In this regard Shepard, H. M., et al, *Nature* (1981) 294:563–565 describe a mutein of IFN-β in which the cysteine at position 141 of its amino acid sequence (there are three cysteines in native human IFN-β at positions 17, 31, and 141, Gene (1980) 10:11–15 and *Nature* (1980) 285:542–547) is replaced by tyrosine. This mutein was made by bacterial expression of a hybrid gene constructed from a partial IFN-β cDNA clone having a G→A transition at nucleotide 485 of the IFN-β gene. The mutein lacked the biological activity of native IFN-β leading the authors to conclude that the replaced cysteine was essential to activity.

Directed mutagenesis techniques are well known and have been reviewed by Lather, R. F. and Lecoq, J. P. in *Genetic Engineering* Academic Press (1983) pp 31–50. Oligonucleotide-directed mutagenesis is specifically reviewed by Smith, M. and Gillam, S. in *Genetic Engineering: Principles and Methods,* Plenum Press (1981) 3:1–32.

DISCLOSURE OF THE INVENTION

One aspect of the invention is a synthetic mutein of a biologically active protein which protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutein having at least one of said cysteine residues deleted or replaced by another amino acid.

Another aspect of the invention relates to synthetic structural genes having DNA sequences that have been specifically designed ("designer genes") to encode the above described synthetic muteins. Subaspects of this aspect are expression vectors that include such structural designer genes, host cells or organisms transformed with such vectors, and processes for making the synthetic mutein by culturing such transformants or their progeny and recovering the mutein from the culture. In the case of muteins that have therapeutic utility, therapeutic compositions that contain therapeutically effective amounts of the muteins and therapeutic methods are other aspects of the invention.

Another aspect of the invention is a method of preventing a protein having one or more cysteine residues that is free to form an undesirable disulfide link from forming such a link comprising mutationally altering the protein by deleting the cysteine residue(s) or replacing them with other amino acids.

Still another aspect of the invention is a method for making the above described synthetic structural gene by oligonucleotide-directed mutagenesis comprising the following steps:

(a) hybridizing single-stranded DNA comprising a strand of a structural gene that encodes the parent protein with a mutant oligonucleotide primer that is complementary to a region of the strand that includes the codon for the cysteine to be deleted or replaced or the antisense triplet paired with the codon, as the case may be, except for a mismatch with that codon or antisense triplet, as the case may be, that defines a deletion of the codon or a triplet that encodes said other amino acid;

(b) extending the primer with DNA polymerase to form a mutational heteroduplex; and

(c) replicating the mutational heteroduplex.

The mutant oligonucleotide primers used in this process are another aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram of the amino acid sequence of IFN-β.

FIG. 2 is a schematic illustration showing the preparation of a mutant IFN-β gene by oligonucleotide-directed mutagenesis.

FIG. 3 shows a diagram of plasmid pβ1trp including the IFN-β gene.

FIG. 4 is a diagram of the cloning vector M13mp8 phage.

FIG. 5 shows the restriction map of clone M13-β1.

FIG. 6 shows the sequencing gel pattern of the mutant IFN-β_{ser17} gene showing a single base change in the coding region.

FIG. 7 is a diagram of the expression plasmid pTrp3.

FIG. 8*a* shows the HinfI restriction pattern of clone pSY2501 and FIG. 8*b* shows the resulting two 169bp and 28bp fragments thereof.

FIG. 9 is a restriction map of clone pSY2501.

FIG. 10 shows the coding DNA sequence for the mutein IFN-β_{ser17} with the corresponding amino acid sequence therefor.

FIG. 11 shows the single 18,000 dalton protein band corresponding to IFN-β_{ser17} in the extracts of clones pSY2501 and pβ1trp.

FIG. 12 is a diagram of the plasmid pLW1 which contains the human interleukin-2 (IL-2) gene under the control of the *E.coli* trp promoter.

FIG. 13 is a restriction map of phage clone M13-IL2.

FIG. 14 is a restriction map of the plasmid pLW46.

FIGS. 15*a* and 15*b* show, respectively, the nucleotide sequence of the coding strand of the clone pLW46 and the corresponding amino acid sequence of the IL-2 mutein designated IL-2_{ser125}.

FIG. 16 is a diagram of the plasmid pLW32.

FIG. 17 is a diagram of the plasmid pLW55.

MODES FOR CARRYING OUT THE INVENTION

The present invention provides: muteins of biologically active proteins in which cysteine residues that are not essential to biological activity have been deliberately deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation; mutant genes coding for such muteins; and means for making such muteins.

Proteins that may be mutationally altered according to this invention may be identified from available information regarding the cysteine content of biologically active proteins and the roles played by the cysteine residues with respect to activity and tertiary structure. For proteins for which such information is not available in the literature this information may be determined by systematically altering each of the cysteine residues of the protein by the procedures described herein and testing the biological activity of the resulting muteins and their proclivity to form undesirable intermolecular or intramolecular disulfide bonds. Accordingly, while the invention is specifically described and exemplified below as regards muteins of IFN-β and IL-2 it will be appreciated that the following teachings apply to any other biologically active protein that contains a functionally nonessential cysteine residue that makes the protein susceptible to undesirable disulfide bond formation. Examples of proteins other than IFN-β and IL-2 that are candidates for mutational alteration according to the invention are lymphotoxin (tumor necrosis factor), colony stimulating factor-1, and IFN-α1. Candidate proteins will usually have an odd number of cysteine residues.

In the case of IFN-β it has been reported in the literature and that both the glycosylated and unglycosylated IFNs show qualitatively similar specific activities and that, therefore, the glycosyl moieties are not involved in and do not contribute to the biological activity of IFN-β. However, bacterially produced IFN-β which is unglycosylated consistently exhibits quantitatively lower specific activity than native IFN-β which is glycosylated. IFN-β is known to have three cysteine residues at positions 17, 31 and 141. Cysteine 141 has been demonstrated by Shepard, et al, supra, to be essential for biological activity. In IFN-α, which contains four cysteine residues, there are two intramolecular —S—S— bonds: one between cys 29 and cys 138 and another between cys 1 and cys 98. Based on the homology between IFN-β and IFN-αs cys 141 of IFN-β could be involved in an intramolecular —S—S— bond with cys 31, leaving cys 17 free to form intermolecular crosslinks. By either deleting cys 17 or substituting it by a different amino acid, one can determine whether cys 17 is essential to biological activity, and its role in —SS— bond formation. If cys 17 is not essential for the biological activity of the protein, the resulting cys 17-deleted or cys 17-substituted protein might exhibit specific activity close to that of native IFN-β and would possibly also facilitate isolation and purification of the protein.

By the use of the oligonucleotide-directed mutagenesis procedure with a synthetic oligonucleotide primer that is complementary to the region of the IFN-β gene at the codon for cys 17 but which contains single or multiple base changes in that codon, a designer gene may be produced that results in cys 17 being replaced with any other amino acid of choice. When deletion is desired the oligonucleotide primer lacks the codon for cys 17. Conversion of cys 17 to neutral amino acids such as glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine is the preferred approach. Serine and threonine are the most preferred replacements because of their chemical analogy to cysteine. When the cysteine is deleted, the mature mutein is one amino acid shorter than the native parent protein or the microbially produced IFN-β.

Human IL-2 is reported to have three cysteine residues located at positions 58, 105, and 125 of the protein. As in the case of IFN-β, IL-2 is in an aggregated oligomeric form when isolated from bacterial cells and has to be reduced with reducing agents in order to obtain a good yield from bacterial extracts. In addition, the purified reduced IL-2 protein is unstable and readily reoxidized upon storage to an oligomeric inactive form. The presence of three cysteines means that upon reoxidation, the protein may randomly form one of three possible intramolecular disulfide bridges, with only one of those being the correct bridge as found in the native molecule. Since the disulfide structure of the native IL-2 protein is not known, it is possible to use the present invention to create mutations at codons 58, 105 and 125 of the IL-2 gene and identify which cysteine residues are necessary for activity and therefore most likely to be involved in native disulfide bridge formation. In the same vein, the cysteine residue that is not necessary for activity can be modified so as to prevent the formation of incorrect intramolecular disulfide bridges and minimize the chance of intermolecular disulfide bridges by removal or replacement of the free cysteine residue.

The size of the oligonucleotide primer is determined by the requirement for stable hybridization of the primer to the region of the gene in which the mutation is to be induced, and by the limitations of the currently available methods for synthesizing oligonucleotides. The factors to be considered in designing oligonucleotides for use in oligonucleotide-directed mutagenesis (e.g., overall size, size of portions flanking the mutation site) are described by Smith, M. and Gillam, S., supra. In general the overall length of the oligonucleotide will

be such as to optimize stable, unique hybridization at the mutation site with the 5' and 3' extensions from the mutation site being of sufficient size to avoid editing of the mutation by the exonuclease activity of the DNA polymerase. Oligonucleotides used for mutagenesis in accordance with the present invention usually contain from about 12 to about 24 bases, preferably from about 14 to about 20 bases and still more preferably from about 15 to about 18 bases. They will usually contain at least about three bases 3' of the altered or missing codon.

The method for preparing the modified IFN-β gene broadly involves inducing a site-specific mutagenesis in the IFN-β gene at codon 17 (TGT) using a synthetic nucleotide primer which omits the codon or alters it so that it codes for another amino acid. When threonine replaces the cysteine and the primer is hybridized to the antisense strand of the IFN-β gene, the preferred nucleotide primer is GCAATTTTCAGACTCAG (underlining denotes the altered codon). When it is desirable to delete cysteine, the preferred primer is AGCAATTTTCAGCAGAAGCTCCTG, which omits the TGT codon for cys. When cysteine is replaced by serine, a 17-nucleotide primer, GCAATTTTCAGAGTCAG, which includes an AGT codon for serine is the primer of choice. The T→A transition of the first base in the cys 17 codon results in changing cysteine to serine. It must be recognized that when deletions are introduced, the proper reading frame for the DNA sequence must be maintained for expression of the desired protein.

The primer is hybridized to single-stranded phage such as M13, fd, or ØX174 into which a strand of the IFN-β gene has been cloned. It will be appreciated that the phage may carry either the sense strand or antisense strand of the gene. When the phage carries the antisense strand the primer is identical to the region of the sense strand that contains the codon to be mutated except for a mismatch with that codon that defines a deletion of the codon or a triplet that codes for another amino acid. When the phage carries the sense strand the primer is complementary to the region of the sense strand that contains the codon to be mutated except for an appropriate mismatch in the triplet that is paired with the codon to be deleted. Conditions that may be used in the hybridization are described by Smith, M. and Gillam, S., supra. The temperature will usually range between about 0° C. and 70° C., more usually about 10° C. to 50° C. After the hybridization, the primer is extended on the phage DNA by reaction with DNA polymerase I, T_4 DNA polymerase, reverse transcriptase or other suitable DNA polymerase. The resulting dsDNA is converted to closed circular dsDNA by treatment with a DNA ligase such as T_4 DNA ligase. DNA molecules containing single-stranded regions may be destroyed by S1 endonuclease treatment.

Oligonucleotide-directed mutagenesis may be similarly employed to make a mutant IL-2 gene that encodes a mutein having IL-2 activity but having cys 125 changed to serine 125. The preferred oligonucleotide primer used in making this mutant IL-2 gene when the phage carries the sense strand of the gene is GATGATGCTTCTGAGAAAAGGTAATC. This oligonucleotide has a C→G change at the middle base on the triplet that is paired with codon 125 of the IL-2 gene.

The resulting mutational heteroduplex is then used to transform a competent host organism or cell. Replication of the heteroduplex by the host provides progeny from both strands. Following replication the mutant gene may be isolated from progeny of the mutant strand, inserted into an appropriate expression vector, and the vector used to transform a suitable host organism or cell. Preferred vectors are plasmids pBR322, pCR1, and variants thereof, synthetic vectors and the like. Suitable host organisms are *E.coli*, Pseudomonas, *Bacillus subtilis, Bacillus thuringiensis*, various strains of yeast, *Bacillus thermophilus*, animal cells such as mice, rat or Chinese hamster ovary (CHO) cells, plant cells, animal and plant hosts and the like. It must be recognized that when a host of choice is transformed with the vector, appropriate promoter-operator sequences are also introduced in order for the mutein to be expressed. Hosts may be prokaryotic or eukaryotic (processes for inserting DNA into eukaryotic cells are described in PCT applications Nos. US81/00239 and US81/00240 published Sept. 3, 1981). *E.coli* and CHO cells are the preferred hosts. The muteins obtained in accordance with the present invention may be glycosylated or unglycosylated depending on the glycosylation occurring in the native parent protein and the host organism used to produce the mutein. If desired, unglycosylated mutein obtained when *E.coli* or a Bacillus is the host organism, may be optionally glycosylated in vitro by chemical, enzymatic and other types of modifications known in the art.

In the preferred embodiment of the subject invention respecting IFN-β, the cysteine residue at position 17 in the amino acid sequence of IFN-β, as shown in FIG. 1, is changed to serine by a T→A transition of the first base of codon 17 of the sense strand of the DNA sequence which codes for the mature IFN-β. The site-specific mutagenesis is induced using a synthetic 17-nucleotide primer GCAATTTTCAGAGTCAG which is identical to a seventeen nucleotide sequence on the sense strand of IFN-β in the region of codon 17 except for a single base mismatch at the first base of codon 17. The mismatch is at nucleotide 12 in the primer. It must be recognized that the genetic code is degenerate and that many of the amino acids may be encoded by more than one codon. The base code for serine, for example, is six-way degenerate such that the codons, TCT, TCG, TCC, TCA, AGT, and ACG all code for serine. The AGT codon was chosen for the preferred embodiment for convenience. Similarly, threonine is encoded by any one of codons ACT, ACA, ACC and ACG. It is intended that when one codon is specified for a particular amino acid, it includes all degenerate codons which encode that amino acid. The 17-mer is hybridized to single-stranded M13 phage DNA which carries the antisense strand of the IFN-β gene. The oligonucleotide primer is then extended on the DNA using DNA polymerase I Klenow fragment and the resulting dsDNA is converted to closed circular DNA with T_4 ligase. Replication of the resulting mutational heteroduplex yields clones from the DNA strand containing the mismatch. Mutant clones may be identified and screened by the appearance or disappearance of specific restriction sites, antibiotic resistance or sensitivity, or by other methods known in the art. When cysteine is substituted with serine, the T→A transition, shown in FIG. 2, results in the creation of a new HinfI restriction site in the structural gene. The mutant clone is identified by using the oligonucleotide primer as a probe in a hybridization screening of the mutated phage plaques. The primer will have a single mismatch when hybridized to the parent but will have a perfect match when hybridized to the mutated phage DNA, as indi-

cated in FIG. 2. Hybridization conditions can then be devised where the oligonucleotide primer will preferentially hybridize to the mutated DNA but not to the parent DNA. The newly generated HinfI site also serves as a means of confirming the single base mutation in the IFN-β gene.

The M13 phage DNA carrying the mutated gene is isolated and spliced into an appropriate expression vector, such as plasmid pTrp3, and *E.coli* strain MM294 is transformed with the vector. Suitable growth media for culturing the transformants and their progeny are known to those skilled in the art. The expressed mutein of IFN-β is isolated, purified and characterized.

The following examples are presented to help in the better understanding of the subject invention and for purposes of illustration only. They are not to be construed as limiting the scope of the invention in any manner. Examples 1–11 describe the preparation of a mutein of IFN-β. Examples 12–20 describe the preparation of a mutein of IL-2.

EXAMPLE 1

Cloning of the IFN-β Gene Into M13 Vector:

The use of M13 phage vector as a source of single-stranded DNA template has been demonstrated by G. F. Temple et al, *Nature* (1982) 296:537–540. Plasmid pβ1trp (FIG. 3) containing the IFN-β gene under control of *E.coli* trp promoter, was digested with the restriction enzymes HindIII and XhoII. The M13mp8 (J. Messing, "Third Cleveland Symposium on Macromolecules: Recombinant DNA," Ed. A Walton, Elsevier Press, 143–153 (1981)) replicative form (RF) DNA (FIG. 4) was digested with restriction enzymes HindIII and BamHI, and mixed with the pβ1trp DNA which had previously been digested with HindIII and XhoII. The mixture was then ligated with T_4 DNA ligase and the ligated DNA transformed into competent cells of *E.coli* strain JM 103 and plated on Xgal indicator plates (J. Messing, et al, *Nucleic Acids Res* (1981) 9:309–321). Plaques containing recombinant phage (white plaques) were picked, inoculated into a fresh culture of JM 103 and minipreps of RF molecules prepared from the infected cells (H. D. Birnboim and J. Doly, *Nucleic Acid Res* (1979) 7:1513–1523). The RF molecules were digested with various restriction enzymes to identify the clones containing the IFN-β insert. The restriction map of one such clone (M13-β1) is shown in FIG. 5. Single-stranded (ss) phage DNA was prepared from clone M13-β1 to serve as a template for site-specific mutagenesis using a synthetic oligonucleotide.

EXAMPLE 2

Site-Specific Mutagenesis:

Forty picomoles of the synthetic oligonucleotide GCAATTTTCAGAGTCAG (primer) was treated with T_4 kinase in the presence of 0.1 mM adenosine triphosphate (ATP), 50 mM hydroxymethylaminomethane hydrochloride (Tris-HCl) pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and 9 units of T_4 kinase, in 50 μl at 37° C. for 1 hr. The kinased primer (12 pmole) was hybridized to 5 μg of ss M13-β1 DNA in 50 μl of a mixture containing 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 10 mM β-mercaptoethanol, by heating at 67° C. for 5 min and at 42° C. for 25 min. The annealed mixture was then chilled on ice and then added to 50 μl of a reaction mixture containing 0.5 mM each of deoxynucleoside triphosphate (dNTP), 80 mM Tris-HCl, pH 7.4, 8 mM $MgCl_2$, 100 mM NaCl, 9 units of DNA polymerase I, Klenow fragment, 0.5 mM ATP and 2 units of T_4 DNA ligase, incubated at 37° C. for 3 hr and at 25° C. for 2 hr. The reaction was then terminated by phenol extraction and ethanol precipitation. The DNA was dissolved in 10 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50% sucrose and 0.05% bromophenylblue and electrophoresed on 0.8% agarose gel in the presence of 2 μg/ml of ethidium bromide. The DNA bands corresponding to the RF forms of M13-β1 were eluted from gel slices by the perchlorate method (R. W. Davis, et al, "Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, N.Y., p. 178–179 (1980)). The eluted DNA was used to transform competent JM 103 cells, grown overnight and ssDNA isolated from the culture supernatant. This ssDNA was used as a template in a second cycle of primer extension, the gel purified RF forms of the DNA were transformed into competent JM 103 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

EXAMPLE 3

Site Specific Mutagenesis:

The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is GCAATTTTCAGACTCAG to change codon 17 of the IFN-β gene from one that codes for cysteine to one that codes for threonine.

EXAMPLE 4

Site Specific Deletion:

The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is AGCAATTTTCAGCAGAAGCTCCTG to delete codon 17 of the IFN-β gene.

EXAMPLE 5

Screening And Identification of Mutagenized Plaques:

Plates containing mutated M13-β1 plaques (Example 1) as well as two plates containing unmutated M13-β1 phage plaques, were chilled to 4° C. and plaques from each plate transferred onto two nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2N NaOH, 1.5M NaCl and 0.2% Triton X-100 for 5 min, and neutralized by layering onto filter papers soaked with 0.5M Tris-HCl, pH 7.5 and 1.5M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2×SSC (standard saline citrate), dried and then baked in a vacuum oven at 80° C. for 2 hr. The duplicate filters were prehybridized at 55° C. for 4 hr with 10 ml per filter of DNA hybridization buffer (5×SSC) pH 7.0, 4×Denhardt's solution (polyvinylpyrrolidine, ficoll and bovine serum albumin, 1×=0.02% of each), 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate buffer pH 7.0 and 100 μg/ml of denatured salmon sperm DNA. ^{32}P-labeled probe was prepared by kinasing the oligonucleotide primer with ^{32}P-labeled ATP. The filters were hybridized to 3.5×10^5 cpm/ml of ^{32}P-labeled primer in 5 ml per filter of DNA hybridization buffer at 55° C. for 24 hr. The filters were washed at 55° C. for 30 min each in washing buffers containing 0.1% SDS and decreasing amounts of SSC. The filters were washed initially with buffer containing 2×SSC and the control filters containing unmutated M13-β1 plaques were checked for

the presence of any radioactivity using a Geiger counter. The concentration of SSC was lowered stepwise and the filters washed until no detectable radioactivity remained on the control filters with the unmutated M13-β1 plaques. The lowest concentration of SSC used was 0.1×SSC. The filters were air dried and autoradiographed at −70° C. for 2–3 days. 480 plaques of mutated M13-β1 and 100 unmutated control plaques were screened with the kinased oligonucleotide probe. None of the control plaques hybridized with the probe while 5 mutated M13-β1 plaques hybridized with the probe.

One of the five mutated M13-β1 plaques (M13-SY2501) was picked and inoculated into a culture of JM 103. ssDNA was prepared from the supernatant and double-stranded (ds) DNA was prepared from the cell pellet. The ssDNA was used as a template for the dideoxy-sequencing of the clone using the M13 universal primer. The result of the sequence analysis is shown in FIG. 6, confirming that the TGT cys codon has been converted to an AGT ser codon.

EXAMPLE 6

Expression of Mutated IFN-β in *E.coli:*

RF DNA from M13-SY2501 was digested with restriction enzymes HindIII and XhoII and the 520 bp insert fragment purified on a 1% agarose gel. The plasmid pTrp3 containing the *E.coli* trp promoter (FIG. 7) was digested with the enzymes HindIII and BamHI, mixed with the purified M13-SY2501 DNA fragment, and ligated in the presence of T_4 DNA ligase. The ligated DNA was transformed into *E.coli* strain MM294. Ampicillin resistant transformants were screened for sensitivity to the drug tetracycline. Plasmid DNA from five ampicillin resistant, tetracylcine sensitive clones were digested with HinfI to screen for the presence of the M13-SY2501 insert. FIG. 8*a* shows the HinfI restriction pattern of one of the clones (pSY2501), comparing it with the HinfI pattern of the original IFN-β clone, pβ1trp. As expected, there is an additional HinfI site in pSY2501, cleaving the 197 bp IFN-β internal fragment to a 169 bp fragment and a 28 bp fragment (FIG. 8*b*). A restriction map of the clone pSY2501 is shown in FIG. 9. The complete DNA sequence of the mutant IFN-β gene is shown in FIG. 10 together with the predicted amino acid sequence.

The plasmid designated as clone pSY2501 was deposited with the Agricultural Research Culture Collection (NRRL), Fermentation Laboratory, Northern Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, 1815 North University Street, Peoria, Ill. 60604 on Mar. 30, 1983 and was assigned accession numbers CMCC No. 1533 and NRRL No. B-15356.

Cultures of pSY2501 and pβ1trp, which include progeny thereof, were grown up to an optical density (OD_{600}) of 1.0. Cell free extracts were prepared and the amount of IFN-β antiviral activity assayed on GM2767 cells in a microtiter assay. Extracts of clone pSY2501 exhibited three to ten times higher activity than pβ1trp (Table 1), indicating that clone pSY2501 was either synthesizing more protein exhibiting IFN-β activity or that the protein made had a higher specific activity.

TABLE 1

EXTRACT	ANTIVIRAL ACTIVITY (U/ml)
pSY2501	6×10^5
pBltrp	1×10^5
ptrp3 (control)	30

In order to determine if clone pSY2501 was synthesizing several times more active protein, the extracts of both clones were electrophoresed on a SDS polyacrylamide gel together with a control extract and the gel stained with coomasie blue to visualize the proteins. As shown in FIG. 11, there was only one protein band corresponding to an apparent 18,000 dalton protein that was present in the extracts of clones pSY2501 and pβ1trp but not in the control extract of ptrp3. This protein, which has a molecular weight of about 20,000 daltons but shows a gel migration pattern of an 18,000 dalton protein was previously shown to be IFN-β by purification of this protein from extracts of pβ1trp. Since there is less of this protein in extracts of pSY2501 than in extracts of pβ1trp, the specific activity of the protein in extracts of clone pSY2501 was higher than that of clone pβ1trp.

EXAMPLE 7

The plasmid pSY2501 was transformed into a competent subvariant of *E.coli* strain MM294, designated MM294-1. A sample of the resulting transformant was deposited in the American Type Culture Collection 12301 Parklawn Drive, Rockville, Md. 20852 U.S. on Nov. 18, 1983 under ATCC number 39,517.

EXAMPLE 8

Production of IFN-β_{ser17}:

IFN-β_{ser17} was recovered from *E.coli* that had been transformed to produce IFN-β_{ser17}. The *E.coli* were grown in the following growth medium to an OD of 10–11 at 680 nm (dry wt 8.4 g/l).

Ingredient	Concentration
NH_4Cl	20 mM
K_2SO_4	16.1 mM
KH_2PO_4	7.8 mM
Na_2HPO_4	12.2 mM
$MgSO_4.7H_2O$	3 mM
Na_3 citrate.$2H_2O$	1.5 mM
$MnSO_4.4H_2O$	30 μM
$ZnSO_4.7H_2O$	30 μM
$CuSO_4.5H_2O$	3 μM
L-tryptophan	70 mg/l
$FeSO_4.7H_2O$	72 μM
thiamine.HCl	20 mg/l
glucose	40 g/l
pH control with NH_4OH	

A 9.9 l (9.9 kg) harvest of the transformed *E.coli* was cooled to 20° C. and concentrated by passing the harvest through a cross-flow filter at an average pressure drop of ~110 kpa and steady-state filtrate flow rate of 260 ml/min until the filtrate weight was 8.8 kg. The concentrate (approximately one liter) was drained into a vessel and cooled to 15° C. The cells in the concentrate were then disrupted by passing the concentrate through a Manton-Gaulin homogenizer at 5° C., ~69,000 kpa. The homogenizer was washed with one liter phosphate buffered saline, pH 7.4 (PBS), and the wash was added to the disruptate to give a final volume of two liters. This volume was continuously centrifuged at 12000×g at a 50 ml/min flow rate. The solid was separated from the supernatant and resuspended in four liters PBS con-

taining 2% by wt SDS. This suspension was stirred at room temperature for 15 min after which there was no visible suspended material. The solution was then extracted with 2-butanol at a 1:1 2-butanol:solution volume ratio. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic phase was then separated and evaporated to dryness to yield 21.3 g of protein. This was resuspended in distilled water at a 1:10 volume ratio.

The recovered product was assayed for human IFN-β activity using an assay based on protection against viral cytopathic effect (CPE). The assay was made in microtiter plates. Fifty μl of minimum essential medium were charged into each well and 25 μl of the sample was placed in the first well and 1:3 volume dilutions were made serially into the following wells. Virus (vesicular stomatitus), cell (human fibroblast line GM-2767), and reference IFN-β controls were included on each plate. The reference IFN-β used was 100 units per ml. The plates were then irradiated with UV light for 10 min. After irradiation 100 μl of the cell suspension (1.2×10^5 cells/ml) was added to each well and the trays were incubated for 18–24 hr. A virus solution at one plaque-forming unit per cell was added to each well except the cell control. The trays were then incubated until the virus control showed 100% CPE. This normally occurred 18–24 hr after adding the virus solution. Assay results were interpreted in relation to the location of the 50% CPE well of the reference IFN-β control. From this point the titer of interferon for all samples on the plate was determined. The specific activity of the recovered product was determined to be 5×10^7 U/mg.

EXAMPLE 9

Acid Precipitation And Chromatographic Purification:

The process of Example 8 was repeated except that after extraction and separation of the aqueous and organic phases and mixing of the organic phase with PBS at a volume ratio of 3:1 the pH of the mixture was lowered to about 5 by addition of glacial acetic acid. The resulting precipitate was separated by centrifugation at 10000–17000$\times$g for 15 min and the pellet was redissolved in 10% w/v SDS, 10 mM DTT, 50 mM sodium acetate buffer, pH 5.5, and heated to 80° C. for 5 min.

The solution was then applied to a Brownlee RP-300, 10 μM, "Aquapore" column using a Beckman gradient system. Buffer A was 0.1% trifluoroacetic acid (TFA) in H_2O; buffer B was 0.1% TFA in acetonitrile. Detection was by ultraviolet absorbance at 280 nm. The solvent program was linear gradient of 0% buffer B to 100% buffer B in three hr. Fractions containing highest interferon activities were pooled and the specific activity of the pooled interferon preparation was determined to be 9.0×10^7 to 3.8×10^8 international units per mg protein, as compared to about 2×10^8 U/mg for native IFN-β.

EXAMPLE 10

Biochemical Characterization of IFN-β Ser_{17}:

Amino acid compositions were determined after 24–72 hr timed hydrolysis of 40 μg samples of IFN in 200 μl of 5.7N HCl, 0.1% phenol, at 108° C. Proline and cysteine were determined in the same fashion after performic acid oxidation; in this case, phenol was omitted from the hydrolysis. Tryptophan was analyzed after 24 hr hydrolysis of 400 μl samples in 5.7N HCl, 10% mercaptoacetic acid (no phenol). Analysis was performed on a Beckman 121MB amino acid analyzer using a single column of AA10 resin.

The amino acid composition calculated from representative 24-,48-, 72-hr acid hydrolyses of purified IFN-β Ser_{17} agrees well with that predicted by the DNA sequence of the cloned IFN gene, minus the missing N-terminal methionine.

The amino acid sequence of the first 58 residues from the amino acid terminus of purified IFN was determined on a 0.7 mg sample in a Beckman 890C sequanator with 0.1M Quadrol buffer. PTH amino acids were determined by reverse-phase HPLC on an Altex ultrasphere ODS column (4.6×250 mm) at 45° C. eluted at 1.3 min at 40% buffer B, and 8.4 min from 40–70% buffer B, where buffer A was 0.0115M sodium acetate, 5% tetrahydrofuran (THF), pH 5.11 and buffer B was 10% THF in acetonitrile.

The N-terminal amino acid sequence of IFN-β Ser_{17} determined matches the expected sequence predicted from the DNA sequence, except for the absence of N-terminal methionine.

EXAMPLE 11

Alternative IFN-β_{ser} Production and Purification Process:

E.coli transformed with pSY2501 were grown in the following medium:

Ingredient	Approximate Initial Concentration
Na_3 Citrate.$2H_2O$	3 mM
KH_2PO_4	30 mM
$(NH_4)_2SO_4$	74 mM
$MgSO_4.7H_2O$	3 mM
$MnSO_4.H_2O$	46 μM
$ZnSO_4.7H_2O$	46 μM
$CuSO_4.5H_2O$	1–2 μM
L-tryptophan	350 μM
$FeSO_4.7H_2O$	74 μM
thiamine.HCl	0.002%
glucose	0.5%

Dow Corning Antifoam polypropylene glycol, 25% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

Temperature was maintained at 37 ±1° C., pH at 6.5 ±0.1 with NaOH, and dissolved oxygen at 30% of air saturation. Optical density and residual glucose measurements were taken at 14 hr and at approximately one hr intervals thereafter. Harvest was made when gluclose consumption reached 40±6 g/l (OD at 680 nm=10–11).

The harvested material was concentrated approximately 3-fold by circulating it through a microporous cross-flow filter under pressure. The concentrated cells were diafiltered against deionized water until the harvest material was concentrated 4–5 fold. The cells were then disrupted by passing them through a Manton-Gaulion homogenizer at $\sim4.1-5.5\times10^4$ kpa. After the intial pass SDS-sodium phosphate buffer was added to a final concentration of 2% SDS, 0.08M sodium phosphate and homogenization was continued for one hr. Solid DTT was then added to a final concentration of 50 mM and the homogenizate was heated to 90 ±5° C. for 10 min. The resulting cell suspension was extracted with 2-butanol at a 1:1 2-butanol:suspension volume ratio in a static mixer. The mixture was then centrifuged and the 2-butanol rich phase was collected.

The 2-butanol rich phase was mixed with 2.5 volumes of 0.1% SDS in PBS. Solid DTT was added to a final concentration of 2 mM. The pH of the mixture was adjusted to 6.2 ±0.1 with glacial acetic acid and this mixture was centrifuged. The resulting paste was collected and resuspended in PBS +10% SDS with pH adjustment to 8.5 ±0.1 using 1N NaOH. Solid DTT was added to a final concentration of 100 mM and the suspension was heated to 90 ±5° C. for 10 min. The suspension was then cooled to ~25° C., the pH was adjusted to 5.5 ±0.1 with glacial acetic acid, and the solution was filtered.

The solution was then applied to a Sephacryl S-200 pre column and the fractions containing highest interferon activities were pooled and concentrated by ultrafiltration with a 10 Kdal molecular weight cutoff. The concentrate was oxidized by adding equimolar amounts of protein and iodosobenzoic acid into a reaction vessel containing 2 mM sodium pyrophosphate, 0.1% SDS and 1 mM EDTA. The pH was controlled during oxidation at 9.0 ±0.1 with 0.5N NaoH and adjusted to 5.5 ±0.2 when oxidation was complete. After oxidation the concentrate was again passed through the ultrafiltration unit with a 10 Kdal molecular weight cutoff.

The concentrate was applied to a main Sephacryl S-200 column and the fractions were analyzed by SDS-PAGE to determine those containing no high molecular weight contaminants. Those fractions were pooled and passed through the ultrafiltration unit. The filtered concentrate was then fractionated on a Sephadex G-75 column. SDS-PAGE analysis of the fractions was made to determine those containing no low or high molecular weight contaminants. Those fractions were pooled for desalting.

A Sephadex G-25 column equilibrated with 1 mM NaOH was loaded with the pooled fractions from the Sephadex G-75 column using distilled water adjusted to pH 10.8–11 with 50% NaOH. The purified product was collected as the void volume peak. This desalted, purified IFN-β mutein may be formulated in known manners for therapeutic administration.

Biological Testing of IFN-β_{ser17}:

Antigenic Comparison:

IFN-β_{ser17} was compared antigenically to IFN-β produced from diploid fibroblasts using virus neutralizing tests. A polyvalent antiserum to the diploid fibroblast IFN-β was prepared in rabbits. This antiserum blocked the antiviral activity of both the diploid fibroblast IFN-β and the IFN-β_{ser17} in the virus neutralization tests, indicating the two proteins are indistinguishable antigenically.

Antiviral Activity:

The purified IFN-β_{ser17} was compared in its antiviral activity to naturally produced IFN-β. Inhibition of vesicular stomatitis virus replication in diploid foreskin fibroblast (HS27F) was indistinguishable from that of the natural molecule. Similarly, inhibition of herpes simplex virus type 1 in HS27F fibroblasts by the natural and mutant proteins were comparable.

Antiproliferative Activity:

The antiproliferation activity of IFN-β_{ser17} for continuous cell lines was compared with that of naturally produced IFN-β. T24 cells derived from a transitional cell carcinoma were treated with 200 units/ml of the proteins. Cell growth was inhibited significantly ($p<0.02$) by both proteins.

Natural Killer (NK) Cell Stimulation:

The ability of IFN-β_{ser17} to stimulate NK cell (spontaneous cell mediated cytotoxicity) activity was tested. Ficoll-hypaque separated peripheral human mononuclear cells (PMC) or NK-enriched lymphocyte preparations (depleted of monocytes by plastic adherence and of OKT3-positive T cells by treatment with OKT3 antibody plus complement) were incubated overnight in growth medium containing various concentrations of IFN-β_{ser17}. ^{51}Cr-labeled target cells were incubated with the effector cells (effector cell:target cell ratio=50:1) for 2–4 hours. NK cell cytoxicity was determined by measuring the amount of label released into the medium. The results of these tests are reported in Table I below.

TABLE I

Target Cell	Effector Cells	NK Cell Cytotoxicity by Interferon (specific % ^{51}Cr release ± SEM) IFN (units/ml) 0	10	30	100	300	1000
T24	PMC	7.23 ± 5.1	23.1 ± 4.4	24.4 ± 1.1	34.1 ± 2.5	50.0 ± 2.0	40.4 ± 4.4
Chang	PMC	4.7 ± 0.5	7.2 ± 0.8	9.5 ± 1.7	15.9 ± 1.3	21.9 ± 1.4	26.9 ± 1.8
Chang	NK Enr	19.2 ± 4.6	39.4 ± 4.1	ND	54.2 ± 6.1	ND	41.7 ± 5.5
K562	NK Enr	41.0 ± 4.6	48.4 ± 3.6	ND	62.2 ± 3.5	ND	63.2 ± 3.5

As shown the target cells were killed more effectively by the IFN-β_{ser17}-treated cells than by the untreated cells.

Clinical Trials:

Phase I clinical trials to verify the safety of IFN-β_{ser17} in humans have been initiated. These trials involve administering the protein to patients intramuscularly and intravenously at doses ranging between 1×10^5 units (1 μg of protein) to 400×10^6 units. In initial phase I clinical trials no unexpected adverse effects have occurred.

As indicated above, the IFN-β_{ser17} preparation exhibits specific activity levels very close to or better than that of native IFN-β. IFN-β_{ser17} has no free sulfhydryl groups but indicates one —S—S— bond between the only remaining cysteines at positions 31 and 141. The protein does not readily form oligomers and appears to be substantially in the monomeric form. The IFN-β_{ser17} obtained in accordance with this invention may be formulated either as a single product or mixtures of the various forms, into pharmaceutically acceptable preparations in inert, nontoxic, nonallergenic, physiologically compatible carrier media for clinical and therapeutic uses in cancer therapy or in conditions where interferon therapy is indicated and for viral infections such as herpes simplex virus I and II, hepatitis B virus, common cold viruses, and rhinovirus. Such media include but are not limited to distilled water, physiological saline, Ringer's solution, Hank's solution and the like. Other nontoxic stabilizing and solubilizing additives such as dextrose, HSA (human serum albumin) and the like may be optionally included. The therapeutic formulations may be administered orally or parenterally such as intravenous, intramuscular, intraperitoneal and subcutaneous

administrations. Preparations of the modified IFN-β of the present invention may also be used for topical applications in appropriate media normally utilized for such purposes. The IFN-β mutein may be administered either locally or systemically by itself or in combination or conjunction with other therapeutic agents such as acyclovir for prophylactic or therapeutic purposes, for example, U.S. Pat. No. 4,355,032. The dose of mutein administered to human patients will depend on whether it is administered continuously (including intermittant) or as a bolus. The amounts administered continuously will typically be lower than the amounts administered as a bolus. The amount will usually be in the range of about 1×10^5 to 4×10^8 units, more usually about 1×10^6 to 1×10^7 units.

The principal advantages of the above described mutein of IFN-β lie in the elimination of a free sulfhydryl group at position 17 in IFN-β, thereby forcing the protein to form correct disulfide links between cys 31 and cys 141 and to assume the conformation ostensibly required for full biological activity. The increased specific activity of the IFN-β_{ser17} enables the use of smaller dosages in therapeutic uses. By deleting the cysteine at position 17 and eliminating the free —SH group, the IFN-β_{ser17} protein does not form dimers and oligomers so readily as the microbially produced IFN-β. This facilitates purification of the protein and enhances its stability.

EXAMPLE 12

The nucleotide sequence for a cDNA clone coding for human IL-2, procedures for preparing IL-2 cDNA libraries, and screening same for IL-2 are described by Taniguchi, T., et al, *Nature* (1983) Vol 24, p 305 et seq.

cDNA libraries enriched in potential IL-2 cDNA clones were made from an IL-2 enriched mRNA fractions obtained from induced peripheral blood lymphocytes (PBL) and Jurkat cells by conventional procedures. The enrichment of the mRNA for IL-2 message was made by fractionating the mRNA and identifying the fraction having IL-2 mRNA activity by injecting the fractions in *Xenopus laevis* oocytes and assaying the oocyte lysates for IL-2 activity on HT-2 cells (J. Watson, *J Exp Med* (1979) 150:1570–1519 and S. Gillis et al, *J Immun* (1978) 120:2027–2032.)

EXAMPLE 13

Screening and Identification of IL-2 cDNA Clones:

The IL-2 cDNA libraries were screened using the colony hybridization procedure. Each microtiter plate was replicated onto duplicate nitrocellulose filter papers (S & S type BA-85) and colonies were allowed to grow at 37° C. for 14–16 hr on L agar containing 50 μg/ml ampicillin. The colonies were lysed and DNA fixed to the filter by sequential treatment for 5 min with 500 mM NaOH, 1.5M NaCl, washed twice for 5 min each time with 5× standard saline citrate (SSC). Filters were air dried and baked at 80° C. for 2 hr. The duplicate filters were pre-hybridized at 42° C. for 6–8 hr with 10 ml per filter of DNA hybridization buffer (50% formamide, 5×SSC, pH 7.0, 5×Denhardt's solution (polyvinylpyrrolidine, plus ficoll and bovine serum albumin; 1×=0.2% of each), 50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 20 μg/ml Poly U, and 50 μg/ml denatured salmon sperm DNA.

A ^{32}P-labeled 20-mer oligonucleotide probe was prepared based on the IL-2 gene sequence reported by Taniguchi, T., et al, supra. The nucleotide sequence of the probe was GTGGCCTTCTTGGGCATGTA.

The samples were hybridized at 42° C. for 24–36 hr with 5 ml/filter of DNA hybridization buffer containing the ^{32}P oligonucleotide probe. The filters were washed two times for 30 min each time at 50° C. with 2×SSC, 0.1% SDS, then washed twice with 1×SSC and 0.1% SDS at 50° C. for 90 min, air dried, and autoradiographed at −70° C. for 2 to 3 days. Positive clones were identified and rescreened with the probe. Full length clones were identified and confirmed by restriction enzyme mapping and comparison with the sequence of the IL-2 cDNA clone reported by Taniguchi, T., et al, supra.

EXAMPLE 14

Cloning of Il-2 Gene into M13 Vector:

The IL-2 gene was cloned into M13mp9 as described in Example 1 using the plasmid pLW1 (FIG. 12) containing the IL-2 gene under the control of the *E.coli* trp promoter. A sample of pLW1 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 U.S. on Aug. 4, 1983 and has been assigned ATCC number 39,405. The restriction map of one clone (designated M13-IL2) containing the IL-2 insert is shown in FIG. 13. Single-stranded phage DNA was prepared from clone M13-IL2 to serve as a template for oligonucleotide-directed mutagenesis.

EXAMPLE 15

Oligonucleotide-directed Mutagenesis:

As indicated previously, IL-2 contains cysteine residues at amino acid positions 58, 105 and 125. Based on the nucleotide sequences of the portions of the IL-2 gene that contain the codons for these three cysteine residues three oligonucleotide primers were designed and synthesized for mutating the codons for these residues to codons for serine. These oligonucleotides have the following sequences.

CTTCTAGAGACTGCAGATGTTTC (DM27) to change cys 58,

CATCAGCATACTCAGACATGAATG (DM28) to change cys 105 and

GATGATGCTCTGAGAAAAGGTAATC (DM29) to change cys 125.

Forty picomoles of each oligonucleotide were kinased separately in the presence of 0.1 mM ATP, 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT and 9 units of T_4 kinase in 50 μl at 37° C. for 1 hr. Each of the kinased primers (10 pmoles) was hybridized to 2.6 μg of ss M13-IL2 DNA in 15 μl of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM $MgCl_2$ and 20 mM β-mercaptoethanol, by heating at 67° C. for 5 min and 42° C. for 25 min. The annealed mixtures were chilled on ice and then adjusted to a final colume of 25 μl of a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM $MgCl_2$, 83 mM NaCl, 17 mM β-mercaptoethanol; 5 units of DNA polymerase I Klenow fragment, 0.5 mM ATP and 2 units of T_4 DNA ligase, incubated at 37° C. for 5 hr. The reactions were terminated by heating to 80° C. and the reaction mixtures used to transform competent JM103 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

EXAMPLE 16

Screening and Identification of Mutagenized Phage Plaques:

Plates containing mutagenized M13-IL2 plaques as well as 2 plates containing unmutagenized M13-IL2 phage plaques, were chilled to 4° C. and phage plaques from each plate were transferred onto two nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2N NaOH, 1.5M NaCl and 0.2% Triton for 5 min, and neutralized by layering onto filter papers soaked with 0.5M Tris-HCl, pH 7.5, and 1.5M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2×SSC, dried and then baked in a vacuum oven at 80° C. for 2 hr. The duplicate filters were pre-hybridized at 42° C. for 4 hr with 10 ml per filter of DNA hybridization buffer (5×SSC, pH 7.0, 4×Denhardts solution (polyvinylpyrrolidine, ficoll and bovin serum albumin, 1×=0.02% of each), 0.1% SDS, 50 mM sodium phosphate buffer, pH 7.0 and 100 μg/ml of denatured salmon sperm DNA. ^{32}P-labelled probes were prepared by kinasing the oligonucleotide primers with labelled ATP. The filters were hybridized to 0.1×10^5 cpm/ml of ^{32}P-labelled primers in 5 ml per filter of DNA hybridization buffer at 42° C. for 8 hr. The filters were washed twice at 50° C. for 30 min each in washing buffers containing 0.1% SDS and 2×SSC, and twice at 50° C. for 30 min each with 0.1% SDS and 0.2×SSC. The filters were air dried and autoradiographed at −70° C. for 2-3 days.

Since the oligonucleotide primers DM28 and DM29 were designed to create a new DdeI restriction site in the mutagenized clones (FIG. 14), RF-DNA from a number of the clones which hybridized with each of these kinased primers were digested with the restriction enzyme DdeI. One of the mutagenized M13-IL2 placques which hybridized with the primer DM28 and has a new DdeI restriction site (M13-LW44) was picked and inoculated into a culture of JM103, ssDNA was prepared from the culture supernatant and dsRF-DNA was prepared from the cell pellet. Similarly, a plaque which hybridized with primer DM29 was picked (M13-LW46) and ssDNA and RF-DNA prepared from it. The oligonucleotide primer DM27 was designed to create a new PstI restriction site instead of a DdeI site. Therefore, the plaques that hybridized to this primer were screened for the presence of a new PstI site. One such phage plaque was identified (M13-LW42) and ssDNA and RF-DNA prepared from it. The DNA from all three of these clones were sequenced to confirm that the target TGT codons for cysteine had been converted to a TCT codon for serine.

EXAMPLE 17

Recloning of the Mutagenized IL-2 Gene for Expression in *E.coli:*

RF-DNA from M13-LW42, M13-LW44 and M13-LW46 were each digested with restriction enzymes HindIII and BanII and the insert fragments purified from a 1% agarose gel. Similarly, the plasmid pTrp3 (FIG. 7) was digested with HindIII and BanII, the large plasmid fragment containing the trp promoter was purified on an agarose gel and then ligated with each of the insert fragments isolated from M13-LW42, M13-LW44 and M13-LW46. The ligated plasmids were transformed into competent *E.coli* K12 strain MM294. The plasmid DNAs from these transformants were analyzed by restriction enzyme mapping to confirm the presence of the plasmids pLW42, pLW44 and pLW46. FIG. 14 is a restriction map of pLW46. When each of these individual clones were grown in the absence of tryptophane to induce the trp promoter and cell free extracts analyzed on SDSpolyacrylamide gels, all three clones, pLW42, pLW44 and pLW46, were shown to synthesize a 14.5 kd protein similar to that found in the positive control, pLW21, which has been demonstrated to synthesize a 14.4 kd IL-2 protein. When these same extracts were subjected to assay for IL-2 activity on mouse HT-2 cells, only clones pLW21 (positive control) and pLW46 had significant amounts of IL-2 activity (Table II below), indicating that cys 58 and cys 105 are necessary for biological activity and changing them to serines (pLW42 and pLW44 respectively) resulted in the loss of biological activity. Cys 125 on the other hand must not be necessary for biological activity because changing it to ser 125 (pLW46) did not affect the biological activity.

TABLE II

Clones	IL-2 Activity (μ/ml)
pIL2-7 (negative control)	1
pLW21 (positive control)	113,000
pLW42	660
pLW44	1,990
pLW46	123,000

FIG. 15*a* shows the nucleotide sequence of the coding strand of clone pLW46. As compared to the coding strand of the native human IL-2 gene clone pLW46 has a single base change of G→C at nucleotide 374. FIG. 15*b* shows the corresponding amino acid sequence of the IL-2 mutein en coded by pLW46. This mutein is designated des-alanyl (ala) IL-2_{ser125}. As compared to native IL-2 the mutein has a serine instead of a cysteine at position 125, has an initial N-terminal methionine (which is processed off), and lacks the initial N-terminal alanine of the native molecule.

A sample of *E.coli* K12 strain MM294 transformed with pLW46 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, U.S. on Sept. 26, 1983 and has been assigned ATCC Number 39,452.

Examples 18 and 19 describe the preparation of an alternative and preferred vector for expressing alanyl (ala) IL-2_{ser125}.

EXAMPLE 18

Construction of Ala-IL-2 Expression Vector pLW32:

A codon (GCG) for alanine was inserted immediately after the initiation codon of the IL-2 gene of pLW1 by oligonucelotide-directed mutagenesis as follows. The oligonucleotide primer, 5'-GAAGTAGGCG-CCATAAG-3', was kinased, hybridized to ssM13-IL2 DNA, and extended using the general procedure of Example 15 to form a mutational heteroduplex. In addition to the insertion of the GCG codon, the mutagenesis generated a new NarI restriction site in the gene. The heteroduplex was converted to closed circular heteroduplex and the circular heteroduplexes were used to transform competent JM103 cells and plated onto agar plates and incubated as in Example 15. The plates were screened to identify mutagenized M13-IL2 by the procedure of Example 16. One mutagenized phage, identified as M13-LW32, was selected for use in additional cloning and RF-DNA was prepared from it. FIG. 16 is a diagram of plasmid pLW32.

EXAMPLE 19

Construction of Ala-IL-2_{ser125} Expressing Clone pLW55:

RF-DNA from M13-LW46 (Examples 16 and 17) was digested with XbaI and PstI and the 530 bp fragment containing the carboxy terminal coding region of the IL-2_{ser125} gene was purified from an agarose gel. Similarly, pLW32 was digested with XbaI and PstI and the large fragment consisting of the plasmid vector and the ala-IL-2 N-terminal coding sequence was purified. The two purified DNA fragments were pooled and ligated using T_4 DNA ligase. The ligated DNA was transformed into competent *E.coli* K-12 strain MM294. Tetracycline resistant transformants were analyzed by restriction enzyme mapping for the presence of a plasmid containing an ala-IL-2_{ser125} gene, identified as pLW55, which has a new DdeI site not found in pLW32. FIG. 17 is a diagram of pLW55. Cell free extracts of bacterial culture containing pLW55 were found to contain over 10^5 units of IL-2 activity per ml by the HT-2 cell assay, J. Watson, supra, and S. Gillis, supra. Ala-IL-2_{ser125} protein is identical to the IL-2_{ser125} molecule shown in FIG. 15*b* except that the former includes the initial N-terminal alanine of the native molecule.

A sample of *E.coli* K-12 strain MM294 transformed with pLW55 was deposited in the American Type Culture Collection on Nov. 18, 1983 and has been assigned ATCC number 39,516.

EXAMPLE 20

Ala-IL-2_{ser125} Production and Purification:

E.coli transformed with pLW55 were grown in a fermenter containing the following medium:

$(NH_4)_2SO_4$	150 mM
KH_2PO_4	21.6 mM
Na_3 Citrate	1.5 mM
$ZnSO_4.7H_2O$	30 μM
$MnSO_4.H_2O$	30 μM
$CuSO_4.5H_2O$	1 μM
pH adjusted to 6.50 with 2.5 N NaOH autoclaved	
Sterile Additions (post autoclave)	
$MgSO_4.7H_2O$	3 mM
$FeSO_4$	100 μM
L-tryptophan	14 mg/l
Thiamine-HCl	20 mg/l
Glucose	5 g/l
Tetracycline	5 mg/l
Ethanol	2%
Casamino acids	2%

Dow Corning Antifoam polypropylene glycol, 20% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

The pH of the fermenter was maintained at 6.8 with 5N KOH. Residual glucose was maintained between 5–10 g/l, dissolved oxygen at 40%, and temperature at 37 ±1° C. The casamino acids (20% stock solution) to a concentration of 2% were added when the OD_{680} was about 10. Harvest was made three hr after the OD reached about 20.

The harvested material was concentrated and homogenized as in Example 11. Following DTT-heat treatment, the material was centrifuged and the resulting paste was extracted with urea to a final concentration of 4M. The suspension was centrifuged and SDS was added to the solid phase to a concentration of 5%. The solution was applied to a Sephacryl S-200 column and fractions containing IL-2 (by SDSPAGE) were pooled. The pooled fractions were applied to a Whatman M-40 column packed with 18 micron Vydac C_4 300 Å pore size bonded phase silica gel equilibrated in 0.1% TFA. The IL-2 mutein was eluted with a gradient of 40% to 60% 2-propanol, containing 0.1% TFA, in 160 min. Fractions containing highest IL-2 activities were pooled and found to have specific activities comparable to native IL-2.

Muteins of IL-2 in which the cysteine at position 125 has been deleted or replaced with another amino acid, such as the mutein IL-2_{ser125} retain IL-2 activity. They may, therefore, be formulated and used in the same manner as native IL-2. Accordingly, such IL-2 muteins are useful for the diagnosis and treatment (local or systemic) of bacterial, viral, parasitic, protozoan and fungal infections; for augmenting cell-mediated cytotoxicity; for stimulating lymphokine activated killer cell activity; for mediating recovery of immune function of lymphocytes; for augmenting alloantigen responsiveness; for facilitating recovery of immune function in acquired immune deficient states; for reconstitution of normal immunofunction in aged humans and animals; in the development of diagnostic assays such as those employing enzyme amplification, radiolabelling, radioimaging, and other methods known in the art for monitoring IL-2 levels in the diseased state; for the promotion of T cell growth in vitro for therapeutic and diagnostic purposes for blocking receptor sites for lymphokines; and in various other therapeutic, diagnostic and research applications. The various therapeutic and diagnostic applications of human IL-2 have been investigated and reported in S. A. Rosenberg, E. A. Grimm, et al, A. Mazumder, et al, and E. A. Grimm and S. A. Rosenberg. IL-2 muteins may be used by themselves or in combination with other immunologically relevent B or T cells or other therapeutic agents: Examples of relevant cells are B or T cells, natural killer cells, and the like and exemplary therapeutic reagents which may be used in combination with the polypeptides of this invention are the various interferons, especially gamma interferon, B cell growth factor, IL-1 and the like. For therapeutic or diagnostic applications, they may be formulated in nontoxic, nonallergenic, physiologically compatible carrier media such as distilled water, Ringer's solution, Hank's solution, physiological saline and the like. Administrations of the IL-2 muteins to humans or animals may be oral or intraperitoneal or intramuscular or subcutaneous as deemed appropriate by the physician. The amount of IL-2 mutein administered will usually range between about 1×10^4 and 2×10^8 units.

Modifications of the above described modes for carrying out the invention that are obvious to those of skill in the fields of genetic engineering, protein chemistry, medicine, and related fields are intended to be within the scope of the following claims.

We claim:

1. Recombinant, synthetic human interferon-β mutein, wherein the cysteine at position 17, numbered in accordance with native interferon-β, is deleted or replaced by a neutral amino acid, and wherein said mutein exhibits biological activity of native, human interferon-β.

2. The synthetic mutein of claim 1 wherein said cysteine residue is replaced by an amino acid selected from the group consisting of serine, threonine, glycine, ala-

nine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan or methionine.

3. The synthetic mutein of claim 1 wherein said cysteine residue has been replaced by an amino acid selected from the group consisting of serine or threonine.

4. The synthetic mutein of claim 1 wherein the mutein is unglycosylated.

5. Biologically active IFN-β_{ser17}.

6. IFN-β_{ser17} as represented by the amino acid sequence represented in FIG. 10.

7. A therapeutic compsition having IFN-β activity comprising a therapeutically effective amount of the synthetic mutein of claims 1, 2, 3, 4, 5, or 6 admixed with a pharmaceutically acceptable carrier medium.

8. A method of regulating cell growth in a patient comprising administering to said patient a cell growth regulating amount of the synthetic mutein of claims 1, 2, 3, 4, 5, or 6.

9. A method of treating a patient for viral disease comprising administering to said patient a viral disease inhibiting amount of the synthetic mutein of claims 1, 2, 3, 4, 5, or 6.

10. A method of stimulating natural killer cell activity in a patient comprising administering to said patient a natural killer cell stimulating amount of the synthetic mutein of claims 1, 2, 3, 4, 5, or 6.

* * * * *

ATTACHMENT B

BETASERON® (brand of Interferon beta - 1b) for Injection
IND 1846 / PLA-92-0495 / ELA-92-0494

April 15, 1983 to April 4, 1986 - The original IND was filed on April 15, 1983 to begin clinical trials with Betaseron in a number of indications. Initially, the trials were designed to demonstrate safety and tolerability of Betaseron at various doses and route of administration in normal subjects and in patients with incurable cancers. Subsequently, phase 1 and 2 trials were initiated in patients with a number of incurable diseases.

Prior to beginning the first MS trial in 1986, trials were conducted to evaluate the efficacy of Betaseron in bladder cancer, breast cancer, head and neck cancer, renal cell carcinoma, colorectal cancer, non-small cell lung cancer, myeloma, lymphoma, melanoma, glioblastoma, multiforme and hairy cell leukemia. In addition to the cancer trials, studies were conducted in non-cancer diseases such as condyloma acuminata, rhinitis and chronic hepatitis B.

April 4, 1986 - Triton Biosciences (TB) submitted first protocol for the indication of multiple sclerosis. A copy of the protocol (TB01-16486) a draft informed consent, FDA Form 1572, and a C.V. for Robert Knobler to conduct this study at Jefferson Medical College in Philadelphia, PA were enclosed.

April 18, 1986 - Cetus had characterized 28 lots of beta-interferon by amino acid composition analysis and over 33 lots by N-terminal sequence analysis. This information amendment described a change in the manufacturing process for beta-interferon.

August 15, 1986 - A new co-investigator, Dr. Arnold Witte, has been added to Dr. Knobler's 1573 for study TB01-16486.

January 16, 1987 - Protocol TB01-16486 amended based on the six weeks interim analysis for the study that indicated safety and efficacy at the 45 million I.U. dose level.

April 15, 1987 - Annual IND progress report submitted.

March 4, 1988 - An ELISA assay developed to test for E. Coli antigens that may remain in the purified IFN-beta solution (G-75 pool). Assay replaces previously used (RIA).

May 6, 1988 - 1988 IND annual report submitted.

June 3, 1988 - Study designs for both the MS and AIDS programs presented during the 12-10-87 and 4-15-88 meetings met with FDA satisfaction in terms of being structured to provide the pivotal data.

August 9, 1988 - Dr. Jeffrey Greenstein conducting study TB01-15486 at Temple University changes his subinvestigator to Dr. Chiara Mangione at Temple.

October 19, 1988 - Support documentation for Dr. J. Greenstein at Temple U. to implement Amendment II for protocol TB 01-16486. Protocol now amended to extend duration of therapy from 108 weeks to 168 weeks.

December 9, 1988 - Support documentation for Dr. J. Greenstein to implement Amendment III to study TB 01-16486 at Temple U.

February 17, 1989 - Dr. Greenstein has replaced Dr. Chiara Mangione as subinvestigator in study TB01-16486 at Temple University Hospital with Dr. Mark L. Moster.

June 5, 1989 - 1989 IND progress report submitted.

October 2, 1989 - Betaseron is currently in double-blind Phase II trials for AIDS and multiple sclerosis (MS). Interim data analyses are factored into both of these trials. When interim data

analyses demonstrate significant durable differences between groups, we will meet with reviewers in division to present analysis results and discuss strategy for a Product License Application (PLA) filing. Item identified for review and comment.

October 27, 1989 - Enclosed is information requested by Dr. Anne Goodman during telephone conversation on October 19, 1989. Dr. Goodman asked for references to specific IND sections, page numbers and submission dates that contain information on the safety evaluation studies.

November 13, 1989 - Protocol TB01-16486 was initiated to evaluate the safety of Betaseron in patients with relapsing-remitting multiple sclerosis and to determine an appropriate dose for further studies. After a 24 week dose-ranging phase, all active drug patients were placed on 45 MIU Betaseron. Revised protocol cover pages, a listing of the protocol changes, and a copy of the revised protocol were enclosed.

December 4, 1989 - Protocol TB01-16486 was submitted on April 4, 1986. Amendment I was submitted May 2, 1986, Amendment II was submitted January 16, 1987, Amendment III was submitted October 19, 1988 and Amendment IV was submitted November 13, 1989.

January 18, 1990 - Protocol TB01-1102. Submitted after telephone discussions with Dr. Anne Goodman. The reason for doing this study is to generate pharmacokinetic data to support a PLA for an AIDS or Multiple Sclerosis (MS) indication.

February 16, 1990 - Notify FDA about a change in the manufacturing process, specifications and building used for the manufacture of Betaseron placebo.

Protocol TB01-1102 submitted 1/18/90 approved by IRB.

April 12, 1990 - A follow-up to the telephone discussion between Mary Levins of Triton and Dr. Anne Goodman of CBER IND Division on March 26, 1990 is provided for comment and approval as a proposed amendment to our Phase III

Betaseron multiple sclerosis (MS) study and a concept sheet describing the mechanism for continuing treatment of patients completing two years on this study. Presented is background information and a brief status report on the Phase III study followed by highlights of the rollover study and protocol amendment.

April 16, 1990 - Protocol TB01-1102 was submitted on January 18, 1990. The protocol has been amended to permit evaluation of Betaseron serum through concentrations after each subcutaneous dose.

May 16, 1990 - The FDA is notified about a change in the location of the manufacturing process for Betaseron final product.

The FDA is notified about the addition of an alternative manufacturer for Betaseron placebo: Sterling Drug Inc.

May 24, 1990 - As a follow-up to our IND submission dated April 17, 1990 and a conference telephone call on May 8, 1990 we provided the finalized protocol amendment to the Betaseron multiple sclerosis study (designated TB01-3586).

July 6, 1990 - As a follow-up to IND submission dated April 12, 1990 and a conference telephone call on May 8, 1990, we are providing the finalized protocol for a new Betaseron Multiple Sclerosis study.

July 17, 1990 - Protocol TB01-35686 was originally submitted on June 3, 1988, with Amendments I and II submitted on March 13, 989 and May 24, 1990, respectively. This Phase III multiple sclerosis study has a total of eleven sites, seven in the United States and four sites in Canada.

Due to an oversight, the appropriate study information for the Canadian sites was never submitted. To correct this we submitted as an amendment to the above referenced IND.

July 26, 1990 - Protocol TB01-35686 was submitted on June 3, 1988, with Amendment I submitted on March 13, 1989 and

Amendment II submitted on May 24, 1990. The changes made to the above protocol and submitted as Amendment I have been approved by the Human Volunteers Research Committee at the University of Maryland in Baltimore.

September 25, 1990 — In previous discussions with the FDA, the Agency had requested that the reproductive toxicity of Betaseron be determined and addressed in a PLA for multiple sclerosis (MS). In this regard, Triton is preparing to initiate a reproductive teratology study in November at the California Primate Research Center, University of California at Davis.

October 26, 1990 - Protocol TB01-35686 was submitted on June 3, 1988 with Amendment I submitted on March 13, 1989 and Amendment II submitted on May 24, 1990. Amendment II has been approved.

November 26, 1990 — On May 24, 1990 Triton amended the protocols for TB01-35686 and TB01-35886, Phase III studies treating multiple sclerosis (MS) with Betaseron, to allow for an interim analysis of the safety and efficacy data from both studies through July 31, 1990.

November 27, 1990 — Notice to FDA that Triton Biosciences Inc. pharmaceutical business was purchased on November 6, 1990. In conjunction with the sale, Triton's name has been changed to Berlex Laboratories, Inc. All correspondence regarding BB-IND 1846 is to be directed to Don Gay.

January 8, 1991 - This submission was in response to FDA letter dated September 11, 1990 regarding study TB01-3103 titled "Double-Blind Placebo-Controlled Phase III Study to Evaluate Long-Term Subcutaneous Betaseron Therapy in Relapsing-Remitting Multiple Sclerosis" (protocol submitted July 6, 1990). FDA questions/observations and Berlex comments presented.

April 2, 1991 - Protocol TB01-3103 was originally submitted on July 6, 1990 with Amendment I filed on September 28, 1990, the protocol was been amended.

Changed the sponsor's name from Triton Biosciences Inc. to Berlex Laboratories, Inc.

June 3, 1991 - 1990 IND annual report submitted.

July 23, 1991 - Protocol TB01-3103 was originally submitted on July 6, 1990 with Amendment I filed on September 28, 1990 and Amendments II, III, and IV filed on April 2, 1991. Another principal investigator has been added.

September 23, 1991 This communication was in regard to a previous submission dated July 23, 1991 and to a telephone conversation with Doug Roberts, M.D. regarding the submission.

November 6, 1991 - Reference is made to our PLA submission meeting request dated November 26, 1990 and the Agency's response dated February 12, 1991. Reference is also made to a meeting between Berlex, Cetus and the Agency on October 25, 1991 and to conference call with Berlex and Doug Roberts, M.D. on October 30, 1991.

Cetus and Berlex now agree that Cetus will be the sole sponsor of the PLA and ELA for Betaseron.

December 23, 1991 Reference is made to our pre-PLA meeting request submission dated November 6, 1991 and to a subsequent telephone conversation with Douglas Roberts, M.D. on November 13, 1991. In response to our request, Dr. Roberts stated that the Agency would need a summary of the final analysis of the two multiple sclerosis (MS) clinical trials (TB01-35686 and TB01-35886) prior to establishing a meeting.

A summary of the clinical trials data from both studies through 2 years is enclosed.

Based on these data, Cetus (in conjunction with Berlex) is planning to file a PLA and ELA for Betaseron in the treatment of MS. We are, therefore, requesting a pre-PLA meeting with the Agency to discuss such an application.

February 13, 1992	-	Submission, in support of the submission dated 12/23/91 and the proposed Pre-PLA meeting of 2/28/92, of the magnetic resonance imaging (MRI) patient evaluations.
March 2, 1992	-	Submission of the slides presented by Berlex and Chiron at the 2/28/92 Pre-PLA meeting.
March 31, 1992	-	Submission to confirm Berlex and Chiron's understanding of issues raised by FDA at the pre-PLA meeting and our plans to address these issues. The letter is divided into the Clinical, Non-clinical and Manufacturing issues. Each significant issue raised by FDA is addressed.
April 13, 1992	-	Submission of Amendment V for Protocol TB01-3103 extending the study an additional year.
May 20, 1992	-	Doug Roberts, IND contact, CBER, called requesting clarification regarding the April 13 submission of Amendment V for protocol TB01-3103.
June 3, 1992	-	D. Redhair, Berlex called Doug Roberts, IND reviewer, CBER as follow-up to M. Levins conversation with Dr. Roberts on 5/20/92. Dr. Roberts finished the conversation by requesting that a clarification letter be submitted to the IND.
June 15, 1992	-	Submission, per Doug Roberts' (IND Reviewer) request, to clarify that Amendment V to TB01-3103 is not unblinding the study.
September 21, 1992		Submission of copy of the revised 1992 Investigator's Brochure with a table listing significant changes made to Brochure.
September 25, 1992		Letter from HPB, Bureau of Biologics, acknowledging receipt of the September 21, 1992 submission of the 1992 Betaseron Investigator's Brochure.
October 20, 1992	-	Notification of address change of Berlex Laboratories from Alameda to Richmond, CA.

November 5, 1992 - Letter from HPB, Bureau of Biologics acknowledging receipt of Berlex letter dated 10/22/92 informing the Bureau of the change in address from Alameda to Richmond, CA.

February 25, 1993 Submission of new protocol BL01-3110 which allows patients from TB01-35686 and TB01-3103 to enter to determine long-term safety of 45mIU Betaseron, given subcutaneously every other day to subjects with multiple sclerosis.

March 25, 1993 - Confirmation of receipt by HPB, Bureau of Biologics of our 2/25/93 submission of protocol BL01-3110, a long term safety study of Betaseron in patients with MS that were previously enrolled in Canadian studies TB01-35886 and TB01-3104.

April 12, 1993 - Submission of form FDA 15782 and curriculum vitaes of two investigators who will be participating in a new study (BL01-3110).

April 13, 1993 - Dr. Kathy Peterson, Chief, CNS Products, HPB was contacted by Donald Gay regarding meeting with her to update HPB on the status of the clinical trials and when we anticipated filing a marketing application for Betaseron.

April 15, 1993 - Ms. Patti McKnight and Mrs. Daryl Krepps, HPB, were contacted by Donald Gay to set up a pre-NDA meeting with them at the request of Dr. Peterson (see telephone report 4/13/93).

April 29, 1993 - Submission of documentation for two new investigators to participate in study BL 01-31100.

May 6, 1993 - Submission providing specimens of vial and carton labeling for open label study BL01-3110 to HPB.

May 6, 1993 - Acknowledgment of receipt of documentation for Dr. Francis and Lapierre to participate in BL01-3110.

May 20, 1993 - Submission to FDA of appropriate documentation for Pierre Duquette, M.D., Montreal, Quebec, Canada to participate in BL01-3110.

May 21, 1993 - Submission of new protocol BL01-3108 entitled "Assessment of Reproductive Function in Women with Multiple Sclerosis Who are Receiving Long-Term Treatment with Betaseron.

June 16, 1992 - The Product License Application (PLA) for Betaseron was submitted to CBER on June 16, 1992. Indicated that the Establishment License (which Chiron holds) would be amended after construction is completed in the Process Development Unit at Chiron. Berlex was identified in the application cover letter as being responsible for preclinical and clinical issues. Chiron was designated as Responsible Head.

June 17, 1992 - Sandra Patterson, Chiron, placed a three-way conference call to Drs. Cavagnaro and Moraseth to discuss and establish a dose for the revised fertility protocol.

July 7, 1992 - Lloyd Johnson, Product Certification, CBER contacted Sandra Patterson, Chiron and requested additional desk copies of the PLA.

July 9, 1992 - Submission, per Dr. Lloyd Johnson's request.

July 21, 1992 - Submission, per Dr. Lloyd Johnson's request.

July 23, 1992 - Bernardita Mendez, Chiron received the PLA reference number for the PLA submitted for Betaseron.

August 5, 1992 - Submission of one copy of PLA volumes 2 and 3 (CMC Technical Summary) at the request of Dr. Lloyd Johnson during the 8/4/92 telephone contact.

August 11, 1992 - Sandra Patterson, Chiron contacted Dr. Lloyd Johnson, Product Certification to determine the status of the FDA review of the proposed exacerbation analysis plan submitted April 14, 1992.

August 13, 1992 - Submission, per Dr. Lloyd Johnson's telephone contact of 8/13/92 with Sandra Patterson, Chiron, of one copy of PLA Volumes 15-21 (CMC Batch Production Records).

August 26, 1992	-	Dr. Lloyd Johnson requested submission ASAP of the following: 1) the teratology study final report (TBT01-31)_ and a Gantt chart for the conduct of the rhesus monkey fertility study (BLT 01-32).
August 27, 1992	-	Submission, per Dr. Lloyd Johnson on August 26, 1992.
August 28, 1992	-	Submission, per Dr. Lloyd Johnson's request on August 28, 1992.
August 31, 1992	-	Submission, per Dr. Lloyd Johnson's request, of the Gantt chart for the conduct of the rhesus monkey fertility study.
September 2, 1992		Dr. Lloyd Johnson (CBER) contacted Sandra Patterson (Chiron) to request additional copies of volumes from the Betaseron PLA.
September 3, 1992		Teleconference call between Berlex and CBER to discuss the location in the PLA of the statistical methodology.
September 14, 1992		Lloyd Johnson of FDA, contacted Bernardita Mendez, Chiron with questions and requests regarding the Betaseron PLA.
September 17, 1992		Sandra Patterson (Chiron) contacted Dr. Lloyd Patterson, CBER, to advise him of readiness for an ELA inspection in May 1993.
September 21, 1992		Berlex called CBER to discuss the request for pivotal trial raw data in disk format.
September 24, 1992		Submission of responses to questions concerning clinical issues posed by the Agency in a telephone conversation between Dr. Lloyd Johnson and Dr. Bernadita Mendez, Chiron Corporation on 9-14-92.

September 25, 1992		Sandra Patterson (Chiron) contacted Dr. Lloyd Patterson CBER to inform him that the response to questions (refer to 9-14-92) had been sent.
October 6, 1992	-	Dr. Sandra Patterson, Chiron and Dana Redhair, Berlex, called Dr. Lloyd Johnson CBER and Dr. Tiwari to reply to Dr. Johnson's questions.
October 9, 1992	-	Submission of one copy each of volumes 6, 7 and 8 of the PLA requested by Dr. Lloyd Johnson, CBER on 10/8/92.
October 14, 1992	-	Sandra Patterson, Chiron contacted Dr. Lloyd Johnson CBER, to determine the status of FDA review of the additional statistical analysis of the pivotal clinical trials data.
October 20, 1992	-	A conference call was set up with participants representing CBER, CDER, Chiron, and Berlex to address FDA's questions.
October 21, 1992	-	Telephone conversation between Dr. Sandra Patterson, Chiron and Dr. Lloyd Johnson, FDA, CBER reiterating the agreements made.
October 26, 1992	-	Submission of the pivotal trial efficacy and programs on diskette.
October 28, 1992	-	Dr. Lloyd Johnson, CBER, contacted Mr. Dana Redhair, Berlex, to state that the Agency would like to replicate the entire MRI procedure to validate our PLA data.
October 30, 1992	-	D. Redhair, Berlex returned the call of Dr. Lloyd Johnson, CBER.
November 2, 1992	-	Submission of one copy each of volumes 27-33 and volume 37 of the PLA requested by Dr. Lloyd Johnson.

November 3, 1992 - A telephone conference call was set up with CBER, CHIRON and Berlex.

November 5, 1992 - Submission of responses to questions posed by the Agency during a telephone conference call on 10/20/92.

November 6, 1992 - Submission of amendment to the PLA presenting the results of a 52 patient subgroup that was examined by MRI scanning every six weeks for two years.

November 9, 1992 - Submission of seven copies of volume 3, pages 178 to 215 of the PLA requested by Dr. Lloyd Johnson.

November 10, 1992 - Dr. Sandra Patterson, Chiron contacted Dr. Lloyd Johnson, CBER.

November 12, 1992 - Submission of the analysis of exacerbation intervals requested by Dr. J. Tiwari, CBER.

November 13, 1992 - Dr. Andrew Larner, PLA Chairman, DCB, CBER was contacted by Dr. Sandra Patterson, Chiron.

November 23, 1992 - Dr. Lloyd Johnson, CBER, contacted Dr. Sandra Patterson, Chiron.

November 24, 1992 - Dr. Joy Cavagnaro, CBER, was contacted by Dr. Sandra Patterson, Chiron.

December 14, 1992 - Submission of center-by-center analyses of the pivotal trial clinical data requested by Dr. Lloyd Johnson, CBER.

December 20, 1992 - Dr. Doug Roberts called Don Gay, Berlex to request 24 copies of the TB01-16486, TB01-35686/35886 protocols.

December 30, 1992 - Berlex and Chiron contacted Dr. Jawahar Tiwari, Division of Biostatistic and Epidemiology, CBER, FDA to provide

answers to Dr. Tiwari's questions (12/29/92 telephone contact).

January 7, 1993 - Submission of interim efficacy report on the third year of data from the pivotal MS clinical trials.

January 8, 1993 - Faxed memo to Dr. S. Patterson, Chiron from Tiwari and Rouzer-Kammeyer, FDA requesting additional data.

January 12, 1993 - Various questions and comments from FDA regarding manufacturing and stability data presented in the PLA.

January 22, 1993 - Mike Bernstein, Executive Secretary, PCNS (Peripheral and Central Nervous System) Advisory Committee informed Dr. Sandra Patterson, Chiron that an PCNS Advisory Committee has been scheduled on 3/19/93 at the FDA Parklawn Building.

January 28, 1993 - Submission of responses to FDA questions regarding the Betaseron Monkey Fertility Study, the Teratology Study and antibody data in primates.

February 1, 1993 - Dr. Sandra Patterson, Chiron phoned Dr. Lloyd Johnson, CBER. Dr. Johnson reviewed the labeling submitted 11/6/92 and indicated that the labeling was unacceptable.

February 19, 1993 - Notification to PLA that a nonproprietary or proper name for Betaseron has been assigned by the U.S. Adopted Names Council (USAN).

March 9, 1993 - Submission of response to FDA comments and questions of 1/12/93 letter regarding CMC issues.

March 11, 1993 - Submission of requested information (refer to telephone conversation dated 3/9/93) regarding MRI.

March 12, 1993 - Submission of proofs of the proposed Betaseron labels for final container, diluent container, and package carton which have been revised as suggested.

March 16, 1993 - With reference to telephone conversation of 3/16/93 between members of the Division of Neuropharmacological Drug Products, Chiron Corp. and Berlex attachments were submitted to FDA:

March 23, 1993 - Dr. Lloyd Johnson, CBER, was contacted by Mr. Dana Redhair, Chiron. Dr. Johnson stated that he had some items that he would like to follow-up on now that the Advisory Committee Meeting had taken place.

March 25, 1993 - Dr. Bernardita Mendez, Chiron called Dr. Lloyd Johnson, CBER, to discuss the Advisory Committee Meeting.

April 2, 1993 - Summary of the three-year efficacy data and a breakdown of all the drop-outs by reason for discontinuations were provided to Dr. Tiwari, FDA by Dr. Suleman Verjee, Berlex.

April 5, 1993 - Dr. Lloyd Johnson, CBER, contacted Dr. Sandra Patterson, Chiron to know exactly when certain items be submitted to the PLA/ELA:

April 5, 1993 - Submission of two summary tables from the three-year MS study as requested.

April 6, 1993 - Submission to the Betaseron Package Insert.

April 7, 1993 - Dr. Suleman Verjee, Berlex provided Dr. Tiwari, CBER, with a diskette containing two datasets which include the MRI and exacerbation data for the subjects who did not withdraw.

April 8, 1993 - Dr. Blair Fraser, CBER, was contacted by Chiron representatives to review the proposed list of specification.

April 9, 1993	-	Dr. Lloyd Johnson, CBER, contacted Dr. Sandra Patterson, Chiron to request information.
April 13, 1993	-	Comments on Betaseron vial label, carton label and diluent label were received from the Agency.
April 13, 1993	-	Submission of copies of Betaseron interferon beta 1b Summary for Basis of Approval.
April 15, 1993	-	Submission of clinical/statistical report for study TB01-3103/3104.
April 16, 1993	-	Faxed to Dr. Tiwari, FDA were two summary tables that were generated from the 3-year MS study.
April 19, 1993	-	Submission to PLA of summary tables that were generated from the 3-year MS study.
April 20, 1993	-	Submission of additional copy of the Betaseron Phase III clinical trial efficacy and safety report.
April 23, 1993	-	Submission of revised package insert.
April 27, 1993	-	Letter received from CBER, FDA requesting that a commitment letter be submitted which outlines proposals for post marketing clinical studies.
May 4, 1993	-	Comments received from FDA (Dr. Blair Fraser) regarding product specifications for Betaseron.
May 5, 1993	-	Letter received from FDA regarding the reviewing of launch promotional materials which have been submitted.
		Comments received from FDA on package insert.

May 10, 1993 - Drs. S. Patterson and B. Mendez, Chiron contacted Dr. Blair Fraser, FDA to discuss bulk and final product specifications.

May 11, 1993 - Review comments on package insert and patient information received via FAX.

Submission of commitment letter outlining proposals for postmarketing clinical studies to address issues raised by the Agency.

May 12, 1993 - Dr. S. Patterson, Chiron contacted Dr. Lloyd Johnson, CBER. to discuss issues.

May 17, 1993 - Dr Mendez and Mr. Redhair, Chiron contacted Drs. Gerard and Fraser, Div. of Cytokine Biology, CBER, FDA to discuss issues:

Submission of proof of the revised Betaseron labels for final container, diluent container, package carton and shelf package carton for review.

May 19, 1993 - Dr. S. Patterson contacted Dr. Blair Fraser to indicate that we were submitting to the PLA the revised and final product specifications.

May 19, 1993 - Submission of Chiron's commitment to pursue the development of those test methods designated "under development" on the Betaseron Certificate of Analysis.

May 20, 1993 - Submission to the PLA of a copy of the letter sent to the Office of Orphan Drug Products Development on 5/13/93. The letter acknowledges the transfer of ownership, rights and privileges of the orphan drug designation for Betaseron in the treatment of relapsing-remitting multiple sclerosis from Berlex to Chiron.

May 21, 1993 - Dr. Blair Fraser, CBER was contacted regarding bulk and final product specifications.

Mr. John Purvis, CDER was contacted to determine the status of the Betaseron package insert.

Submission of proof of the revised diluent container label for FDA review.

May 24, 1993 - Submission of copies of information provided to Dr. Leber, Division of Neuropharmacological Drug Products, FDA.

May 25, 1993 - Submission of two of the analyses requested during 5/14/4 teleconference call between Berlex/Chiron and Div. of Neuropharmacological Drug Products staff.

May 26, 1993 - Dr L. Johnson, CBER was contacted to discuss certain issues.

May 26, 1993 - Dr. Theresa Gerrard, CBER contacted Dr. S. Patterson, Chiron to discuss revisions the Agency wants to see in the 5/11/93 commitment letter for post-marketing studies.

May 27, 1993 - Ms. D. Parshall, QC Lab, FDA was contacted to determine the status of her review of the revised certificate of analysis.

June 1, 1993 - Fax copy received from Dr. L. Johnson of review comments on the package insert.

June 2, 1993 - Submission of proofs of the revised Betaseron labels for package carton and shelf carton for review.

June 3, 1993 - Submission of commitment letter outlining proposals for postmarketing clinical studies to address issues raised by the Agency concerning the use of Betaseron in the treatment of multiple sclerosis.

Letter received from FDA indicating that CBER has completed their review of our PLA application and has determined it to be approvable.

June 4, 1993 - Dr. L. Johnson, CBER was contacted by Dr. S. Patterson, Chiron to advise him of the status of the outstanding items on the PLA/ELA.

June 8, 1993 - Submission to PLA of copy of letter sent to the Office of Orphan Drug Products Development on 6/8/93. The letter informs the Office of Chiron's intention of exercising orphan drug exclusivity for Betaseron upon arrival.

June 10, 1993 - Dr. S. Patterson, Chiron contacted Dr. L. Johnson, CBER, to discuss the status of the remaining outstanding issues concerning the Betaseron PLA/ELA.

June 14, 1993 - Submission of a preliminary report on the development of an assay to measure aggregates in reconstituted Betaseron.

June 18, 1993 - Submission to the PLA of a revised package insert that addresses the issues raised by the Agency.

June 21, 1993 - Submission of the Betaseron patient information sheet containing photographs of the self-injection procedure.

June 24, 1993 - Dr. S. Patterson, Chiron contacted Dr. L. Johnson, CBER to indicate that the SBA was Federal Expressed to FDA today 6/24 and a Fax copy will also be sent.

June 29, 1993 - Submission made by Dr. S. Verjee, Berlex to Dr. J. Tiwari, CBER of a summary of the two-year efficacy analysis.

June 30, 1993 - Dr. L. Johnson, CBER requested cross referencing information concerning the pharmacokinetic data in the package insert.

Dr. L. Johnson, CBER, requested information specific to the package insert.

Letter submitted to PLA in response to phone call from FDA 6/30/93 regarding certain items in package inserts.

July 1, 1993 - Dr. Lloyd Johnson, CBER indicated that the package insert review was proceeding on high priority.

July 2, 1993 - Submission to the PLA of modified pages of the adverse reaction section of the package insert.

Draft comments received from FDA by Fax on package insert for Betaseron.

July 5, 1993 - Dr. S. Patterson, Chiron contacted Dr. L. Johnson, CBER, had a few additional comments to add to the package insert.

July 7, 1993 - Dr. L. Johnson, CBER was contacted by Dr. S. Patterson, Chiron to advise him that we had reviewed the comments from the Agency on the package insert and were incorporating them into the version that would be delivered by FDA by the end of the week.

July 9, 1993 - Dr. T. Gerrard and Dr. L. Johnson, CBER contacted Dr. S. Patterson, Chiron to advise her of additional changes to the package insert requested by the FDA PLA committee.

July 9, 1993 - Submission of revision of the Betaseron package insert for FDA review.

Dr. L. Johnson, CBER contacted Dr. S. Patterson, Chiron to indicate that he had an additional request concerning the package insert.

July 13, 1993 - Submission to the PLA confirming meeting between CBER and Chiron Corp 7/13/93 to present Chiron's Worldwide Manufacturing Plans for interferon beta-1b.

July 15, 1993 - Submission of top three promotional and advertising pieces for FDA review and approval.

Review comments on package insert (submitted 7/9/93) received from FDA.

July 16, 1993 - Submission to FDA of seven other promotional and advertising pieces for FDA review and approval.

Dr. J. Latts, Mr. D. Gay, Berlex and Dr. S. Patterson, Chiron contacted L. Johnson, CBER, FDA.

Submission of the revised package insert which incorporated the changes requested on 7/15/93.

July 20, 1993 - Comments received from FDA regarding promition material.

Fax received from T. Gerrard, FDA indicating a commitment letter should be sent stating that assays will be developed that will improve the quantitation and characterization of patient antibodies to interferon-beta.

Comments received from FDA on the following promotional labeling.

Submission of the revised Package Insert for Betaseron which incorporated changes requested on 7/19/93.

Commitment made to FDA to develop assays that will improve the quantitation and characterization of patient antibodies to Betaseron.

July 21, 1993 - Submission of the revised Summary Basis of Approval for Betaseron which incorporated the changes most recently made to the package insert.

Submission of revised versions of ten advertising pieces discussed with the Agency 7/21/93.

July 22, 1993 - Submission of the VNS (Video News Release) and B-Roll video which used the script approved by the Agency 7/21/93.

S. Risso, CBER, FDA contacted Dr. S. Patterson, Chiron to discuss the wording in the license letter.

Letter sent to the Agency stating understanding and agreement to the commitments, obligations, and requirements set forth in the accelerated approval for biological products regulations.

July 23, 1993 - Approval letter and product license received which authorizes Chiron Corporation to manufacture and ship for sale, barter or exchange in interstate and foreign commerce interferon-beta 1b for use in ambulatory patients with relapsing-remitting multiple sclerosis to reduce the frequency of clinical exacerbations. Berlex Laboratories, Richmond, CA is cited as the distributor.

Written commitments made to FDA are acknowledged in the letter.

ATTACHMENT C

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 4,588,585
DATED : May 13, 1986
INVENTOR(S) : David F. Mark et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the first (title) page of the specification after the section titled "Related U.S. Application Data" insert the following section:

-- Foreign Application Priority Data

Oct. 10, 1983 Ireland 2380/83--

Signed and Sealed this

Tenth Day of May, 1988

Attest:

Attesting Officer

DONALD J. QUIGG

Commissioner of Patents and Trademarks

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"FEE ADDRESS" INDICATION FORM

COPY OF YOUR INFORMATION

ADDRESS TO:
COMMISSIONER OF PATENTS AND TRADEMARKS
BOX MAINTENANCE FEE
WASHINGTON, D.C. 20231

Please recognize as the "fee address" under provisions of 37 CFR 1.363 the following address;

COMPUTER PACKAGES ANNUITY SERVICE INC.
414 HUNGERFORD DR. SUITE 300
ROCKVILLE, MD 20850

PAYOR NUMBER: 000204

In the following listed application(s) or patent(s) for which the issue fee has been paid.

PATENT NUMBER	SERIAL NUMBER	PATENT DATE	U.S. FILING DATE	OUR REFERENCE	
4588585 ✓	655897	13MAY86	28SEP84	CS2083	2A

359

25297

SIGNED Albert P. Halluin

Typed name of person signing: Albert P. Halluin

Title & Corporation: Vice President & Chief Intellectual Property Couns

(check one)
|___| Owner of record
|_X_| Owner's attorney or agent of record

Any prior fee addressee form previously submitted is hereby revoked.

FORM PTO-1537

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re:	U.S. Patent No. 4,588,585	:
Issued:	May 13, 1986	:
Inventors:	David F. Mark Leo S. Lin Shi-da Yu Lu	:
For:	HUMAN RECOMBINANT CYSTEINE DEPLETED INTERFERON-β MUTEINS	:

TRANSMITTAL LETTER

Honorable Commissioner of
Patents and Trademarks
BOX PATENT EXTENSION
Washington, D.C. 20231

Sir:

Being filed herewith are the following papers:

1. Declaration Under 37 C.F.R. § 1.740(b);
2. Application for Extension of Patent Term Under 35 U.S.C. § 156;
3. Attachment A (copy of U.S. Patent No. 4,588,585);
4. Attachment B (brief description under 37 C.F.R. § 1.755); and
5. Attachment C - a copy of the Certificate of Correction;
6. Attachment D - a copy of the 1st maintenance fee paid - receipt;
7. A certified duplicate of all of the above.
8. Three courtesy working copies of 1-7.

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Authorization is hereby granted to charge the fee of $1000 under 37 C.F.R. § 1.20(j) for fiing of an application for extension of the term of a patent to counsel's Deposit Account No. 13-3402. Two copies of this page are attached for this purpose. Authorization is also granted to charge any other fee which might be necessary in conjunction with this filing.

DATE: 9/9/93

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